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Implications to the Aquatic Environment of Polynuclear Aromatic Hydrocarbons Liberated from Northern Great Plains Coal

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The effects of leaching processes upon Western Great Plains coal was investigated to ascertain the potential impact of the organic components on aquatic organisms. Acute and chronic toxicity testing of coal leachate indicated no lipophilic fraction containing polynuclear aromatic hydrocarbons (PAH) that might be anticipated to bioaccumulate. HPLC-GC analysis indicated that the PAH content was of a comparable concentration to samples obtained from Lake Superior. GC-MS analysis of the lipophilic materials that are adsorbed on the coal particulates indicated that they were predominantly low molecular weight PAH's (i.e., naphthalenes, phenanthrenes, anthracenes, etc), alkanes, and heterocycles. Synthetic methodology was developed to provide standard samples of alkylated PAH's of the type observed during the MS analysis.

The biological studies on PAH's were aided by the use of a combined HPLC-GC analysis procedure (ng/l detection level) developed specifically for this program. The biological investigation resulted in obtaining bioaccumulation factors in the range of 1000-5000 for several PAH's.

Selected PAH's of various structural types were also shown to be quite susceptible to "second-order" anthropogenic transformations such as chlorine disinfection.

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Volatilization		Daphnia pulicaria		06/T
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IMPLICATIONS TO THE AQUATIC ENVIRONMENT OF POLYNUCLEAR AROMATIC HYDROCARBONS LIBERATED FROM NORTHERN GREAT PLAINS COAL

by

Robert M. Carlson, Alan R. Oyler, Ellen Heath Gerhart,
Ronald Caple, Kenneth J. Welch, Herbert L. Kopperman,
Donald Bodenner, and Dale Swanson
Department of Chemistry
University of Minnesota
Duluth, Minnesota 55812

Grant No. R803952-03-1

Project Officer

Douglas W. Kuehl
Physiological Effects of Pollutants Section
Environmental Research Laboratory
Duluth, Minnesota 55804

ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
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FOREWORD

Our nation's fresh waters are vital for all animal and plant life, yet our diverse uses of water -- for recreation, food, energy, transportation, and industry -- can physically and chemically alter lakes, river, and streams. Such alterations threaten terrestrial organisms, as well as the aquatic ones. The Environmental Research Laboratory in Duluth, Minnesota, develops methods, conducts laboratory and field studies, and extrapolates research findings

-- to determine how physical and chemical pollution affects aquatic life;

-- to assess the effects of ecosystems on pollutants;

-- to predict effects of pollutants on the ecosystems through use of models; and

-- to measure the rate of uptake and bioaccumulation of pollutants in aquatic organisms that are consumed by other animals, including man.

A comprehensive program was designed in 1974 because of the "Energy Crisis" of 1973 to study the adverse effects on the aquatic environment being created by new energy sources and technologies. Data being reported in this grant are a part of the comprehensive program and deal with the possible effects of coal storage on aquatic ecosystems.

Acute and chronic toxicity tests were conducted using fish which indicated little if any adverse effect. Bioaccumulation, however, was noted with several polynuclear aromatic hydrocarbons (PAH) in the range of 1000-5000, and that these PAH's induced a mixed-function oxidase (MFO) activity in trout similar to those of other mammalian systems.

These findings will be integrated with other studies to aid elected officials in making environmentally sound decisions on future energy developments.

Donald I. Mount, Ph.D.
Director
Environmental Research Laboratory-Duluth

ABSTRACT

The effects of leaching processes upon Western Great Plains coal were investigated to ascertain the potential impact of the organic components on aquatic organisms. Acute and chronic toxicity testing indicated no critically adverse effects, which led to a subsequent detailed study on the lipophilic fraction containing polynuclear aromatic hydrocarbons (PAH) that might be anticipated to bioaccumulate. HPLC-GC analysis of coal leachate indicated that the PAH content was of a comparable concentration to samples obtained from Lake Superior. GC-MS analysis of the lipophilic materials that are adsorbed on the coal particulates indicated that they were predominantly low molecular weight PAH's (i.e. naphthalenes, phenanthrenes, anthracenes, etc.), alkanes, and heterocycles. Synthetic methodology was developed to provide standard samples of alkylated PAH's of the type observed during the MS analysis.

The biological studies on PAH's were aided by the use of a combined HPLC-GC analysis procedure (ng/ℓ detection level) developed specifically for this program. The biological investigation resulted in obtaining bioaccumulation factors in the range of 1000-5000 for several PAH's and noting that the PAH's that induced mixed-function oxidase (MFO) activity in rainbow trout were those that showed similar effects in mammalian systems.

Selected PAH's of various structural types were also shown to be quite susceptible to chemical transformations during conditions typical to chlorine disinfection. The chlorination products that were observed during the course of this investigation were mixtures of chloro- and oxygenated derivatives.

This report was submitted in fulfillment of Grant # R803952 by the University of Minnesota, Duluth, under the sponsorship of the Environmental Protection Agency. Work was completed as of July 1, 1978.

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ABBREVIATIONS

AH	-- aniline hydroxylase
AHH	-- aryl hydrocarbon hydroxylase
B(a)P	-- benzo(a)pyrene
bp	-- boiling point
GC	-- gas chromatography
GC-MS	-- gas chromatographic mass spectrometry
GPC	-- gel permeation chromatography
HPLC	-- high performance liquid chromatography
IR	-- infrared
MFO	-- mixed-function oxidase
NMR	-- nuclear magnetic resonance
PAH	-- polynuclear aromatic hydrocarbon polycyclic aromatic hydrocarbon
PID	-- photoionization GC detector
TLC	-- thin layer chromatography
UV	-- ultraviolet

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SECTION 1

INTRODUCTION

COAL COMPOSITION

Coal is a compact material which was produced by metamorphosis of plant matter over periods of time up to about 400 million years. This metamorphic process proceeded through a continuous series of alterations[1] (living plants → peat → lignite → subbituminous coal → bituminous coal → anthracite) which resulted in an increase in the relative amount of carbon present (up to 93% for anthracite) and a decrease in the relative amount of volatile matter.[1,2] The composition of the coal includes inorganic mineral matter from the surrounding geological formations and organic matter derived from the remains of the plants.[1] The organic matter is a complex mixture of various macromolecules which varies according to the stage of metamorphosis[1](rank). The structures of these molecules are not well understood although some models have been proposed.[1,3] In addition, polynuclear aromatic hydrocarbons, including 1,2-benzanthracene, 3,4-benzopyrene, benzo-g,h,i-perylene, perylene, phenanthrene, and 1,2-benzopyrene, have been found to be associated with raw coal.[4,5]

PRESENT UTILIZATION OF COAL

The estimated coal reserves in the United States amount to about three trillion tons, which represents about 50% of the total known world coal reserves and about 80% of the total fossil fuel reserves of this country.[6] Western coal, which has a low sulfur content and is frequently close enough to the surface for strip-mining, constitutes at least 72% of the U.S. coal reserves.[7] The estimated coal reserves of the world are capable of providing enough energy for 730 years based on a consumption rate equal to that of 1973.[8] Current U.S. production is around 700 million tons per year[9] and the total world production is around 2000 million tons per year.[10] The major portion of the coal currently produced is burned directly for the generation of electricity and for heating. In this country, about 20%[11] of our energy needs are met by coal. Most of the remaining coal production is carbonized to produce coke which is utilized in steel production as an ore-reducing agent.[10] The volatile byproducts of coke production include[10,12] benzol (consisting mainly of benzene and toluene), tar (consisting mainly of polynuclear aromatic hydrocarbons of bp > 180°C) and gases (consisting mainly of hydrogen and methane).

FUTURE UTILIZATION OF COAL

In the future a dramatically increased usage of coal to replace dwindling supplies of oil and natural gas in the production of energy and chemicals is anticipated.[7-9,11,13-16] However, the exact processes to be employed and the timetable for their introduction have not yet been determined.

Possibilities for energy production from coal include improved methods of direct combustion,[23] combustion of gases[8,11,15,16,24] and liquids[15,16,18,21,24-27] derived from coal, and direct generation of electricity from coal by magnetohydrodynamics.[28,29] Chemical feedstocks[30] such as ammonia,[8] methanol,[8] acetic acid,[8] ethylene glycol,[8] ethylene,[8] benzene-toluene-xylenes[14] (BTX), petrochemicals[14,18,31] and others[14] can also be derived from coal.

ENVIRONMENTAL AND HEALTH CONCERNS

There are considerable concerns about the environmental and health aspects of increased coal utilization because of the types of compounds that are associated with coal and its transformation processes.[9,32-35] These include inorganic compounds such as heavy metals, sulfur dioxide, and nitrogen oxides and organic compounds such as phenols, arylamines, alkanes, mono- and polycyclic aromatic hydrocarbons and their sulfur and nitrogen heterocyclic analogs.

Extensive studies on phenols indicate that, in general, these compounds have a high acute toxicity,[36-38] a low potential for bioaccumulation,[36] and are readily degraded by microbes.[38-40] Arylamines have been found to be toxic to blue-green algae[41] and other aquatic organisms.[40] Alkanes undergo microbial degradation,[42-43] are relatively non-toxic[44-45] and may be accumulated in aquatic organisms.[43,44,46]

The aromatic hydrocarbons and their heterocyclic analogs have caused the most concern. Mutagenicity[47-50] and carcinogenicity[51-53] are associated with many members of this group of compounds including benzo(a)pyrene (B(a)P), benzo(a)anthracenes, and chrysenes. Toxicity has also been reported for some members[51,54,55] such as 6-methylquinoline[37] and phenanthrenes.[45] The bioaccumulation of these compounds may be a problem as well[44,46,54,56] (see Section 6 also).

Metabolic activation of polycyclic aromatic hydrocarbons (PAH) is necessary for cancer production.[57,58] Complex, inducible[53,59] enzyme systems which contain cytochrome P-450[52,53,60] and which are located in the endoplasmic reticulum[61] and the nucleus[62,63] of many mammalian cells (including the cells of liver, skin, blood, placenta and others in humans[64]) convert the PAH's into metabolites such as phenols, epoxides, alcohols, and ketones. Some of the metabolites are known to bind covalently to DNA,[55,56,66-74] RNA,[55,68] and proteins[55,66-68,75] based on studies on benzo(a)pyrene,[68,72] benzo(a)anthracene,[77] quinoline,[50] benzene[70] and other compounds. Epoxides or diol epoxides are currently believed to be the "ultimate carcinogens",[50,69,72-82] but other metabolites such as quinones,[83] phenols,[84] and some intermediate species[53,57] such as radical cations[85] may also deserve consideration.

Extensive studies[68,71,76] on the metabolism of the carcinogen benzo(a)-pyrene indicate that the diastereomeric 7,8-diol-9,10-epoxides may be the "ultimate carcinogens." Covalent bonding of these epoxides with nucleic acids occurs primarily with the 2-amino group of guanine[68,71] (Scheme 1) as well as the 6-amino group of adenine,[71] and the 3-N, 4-amino, or 2-oxo group of cytosine.[71] In addition, the reaction of these epoxides with phosphotriester linkages is known to cause strand scission in DNA[68,86] (Scheme 1). However, final conclusions about the exact mechanism of PAH produced mutagenicity and carcinogenicity cannot be made at present.[68]

POINTS OF ENVIRONMENTAL ENTRY OF COAL-RELATED ORGANIC COMPOUNDS

PAH's and other coal-related compounds may enter the environment at a number of stages along the coal utilization sequence from the initial mining operations to the final waste disposal. Mining operations and proposed underground gasification techniques[87,88] may introduce significant amounts of inorganic[89,90] and organic pollutants including PAH's[91,92] into ground and surface water. Transport of the coal from the mines to the site of utilization by proposed slurry pipelines might also be a problem.[93,94] Storage, which may require up to 50 acres at a conversion site,[95] may introduce pollutants into the environment through dusting emission[95] and runoff of particulates and dissolved materials.[95-98]

Aqueous effluents result from the usage of large quantities of water in many parts of coal conversion processes including pre-treatment of coal, gas scrubbing, cooling, and quenching.[98] The principal types of organic materials in these effluents are phenols and nitrogen containing heterocyclic aromatic compounds but mono- and polycyclic aromatic hydrocarbons may also be present.[35,37,40,99,100]

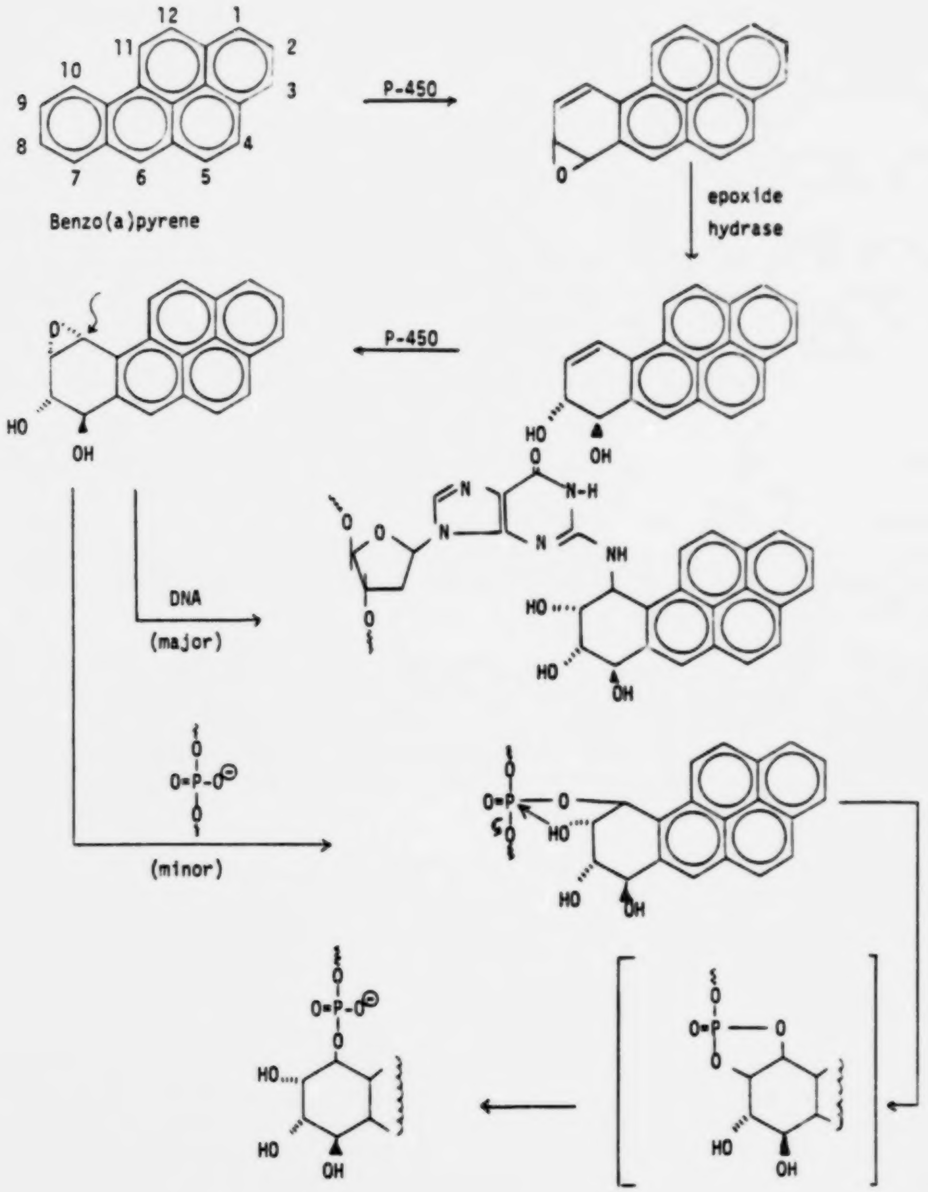
Airborne emissions from coal combustion and conversion processes are believed to be the major source of ariborne polycyclic and heterocyclic aromatic hydrocarbons[35,101-108] in the atmosphere (see Table 9). These emissions have been linked with an increased incidence of human cancer. Moreover, filtrates of respirable coal fly ash have been shown to be mutagenic to *Salmonella typhimurium*.[109]

Finally, the products of coal conversion (i.e., gasification, liquification, etc.) which may contain varying amounts and types of polycyclic compounds, phenols, arylamines, and alkanes may also present environmental and health hazards.[10,100,110-121]

PRESENT WORK

The purpose of the present project was to determine the identity, quantity, and environmental implications of organic compounds liberated from Northern Great Plains coal during storage at facilities such as the one located in Superior, Wisconsin. At this facility coal, transported to Superior by rail from the mines in Montana, is stored in an open-air holding prior to loading on to large lake freighters.

Scheme 1[68,71,77,86]


1
2
3
4
5
6
7
8
9
10
11
12
Benzo(a)pyrene
P-450
epoxide hydrase
P-450
DNA
(major)
(minor)

SECTION 2

CONCLUSIONS

The study of organic leaching from Northern Great Plains coal has shown that solubilized levels less than 10-50 ng/ℓ of an individual polynuclear aromatic hydrocarbon (PAH) will be obtained upon equilibration at 25°. Moreover, the predominant compound types observed in the leachate (e.g. the lower molecular weight PAH's and alkyl PAH's) are not those regarded to be highly carcinogenic and are present at concentrations comparable to background levels of PAH's in Lake Superior.

There are also polar material leached from coal, but neither these compounds nor the PAH's exhibited severe adverse effects on aquatic organisms at the concentrations tested. The lipophilic organic compounds appear to be associated with the particulate coal fraction of leachate but can be efficiently released through volatilization processes (i.e. steam distillation) that are possible in a natural setting.

PAH's typically considered carcinogens and MFO inducers in mammalian systems were found to initiate MFO induction in rainbow trout. Moreover, for the specific carcinogen benzo(a)pyrene, a minimum concentration of about 300 μg/kg in the tissue of rainbow trout was found to be necessary to induce MFO production. All the PAH's studied (dibenzofuran, fluorene, phenanthrene, 9-chlorophenanthrene, β-naphthoflavone, 1-methylphenanthrene, fluoranthene, and pyrene) were readily bioaccumulated (bioconcentration factor ≃ 1000-5000), but the attainment of a steady state value cannot always be assumed due to external influences such as the presence in the water or tissue of other compounds or an enhanced level of MFO activity in the exposed fish. The release or conversion of bioaccumulated PAH's was very rapid, usually occurring in less than 4 days. In addition a study showed the vulnerability of PAH to aqueous chlorination under conditions typical of those used for disinfection. The product types (chloro PAH, chlorohydrins, and quinones) observed during this investigation were those expected not only on mechanistic considerations but also on reports of products isolated from other oxidation processes.

The development of a rapid and selective technique for the general synthesis of alkylated PAH's aided the characterization process. The utility of the synthetic method was demonstrated by the preparation of several polymethyl naphthalenes. The sensitive and versatile high performance liquid chromatography - gas chromatography system was designed for the analysis of PAH's in water and tissue with a lower limit of detection of ≃ 10-50 ng/ℓ for an 18 ℓ sample and about 100 μg/kg for a 1 g sample, respectively.

In summary, the chemical and biochemical portions of the study resulted in the development of new analytical methodology, insight into the susceptibility of PAH's toward external transformations involved in such processes as chlorine disinfection and metabolism, and the tendency of PAH's to bioaccumulate in aquatic organisms.

SECTION 3

RECOMMENDATIONS

The results of the study suggest areas for further chemical investigation before design and siting criteria can be firmly established for coal storage and handling facilities. These areas of study include the analysis of the polar portion of the coal leachate and distillate, the determination of the ability of aquatic organisms to bioaccumulate PAH *from* particulate matter, the examination of environmental samples of water and sediment for the presence of the chlorine disinfection products, and the field evaluation of the impact of the volatilization process.

Field studies of the tissue content of PAH and the levels of MFO activity in fish would be appropriate. Determination of the relative importance of uptake of PAH from food, in comparison to uptake from water and particulates, would be useful for the estimation of maximum bioconcentration potential of PAH in fish. In addition, field studies of the PAH content of organisms of several trophic levels would illuminate the environmental partitioning of PAH within a biological community.

SECTION 4

CHEMICAL AND BIOLOGICAL STUDIES OF NORTHERN GREAT PLAINS COAL

NORTHERN GREAT PLAINS COAL

Northern Great Plains coal is a subbituminous variety of coal which contains[122] 72-76% carbon (mineral-matter free), 18% oxygen, and 35-50% volatile matter. A large portion of the volatile matter is probably water.[123]

Experimental Details

The coal was originally derived from the Decker Mine in Montana and arrived in Superior, Wisconsin, on October 25, 1975. Sixty pounds of coal were taken from each of twelve railroad cars and the combined samples were mixed in a large fiber barrel. The coal was then ground in a Wiley mill and sieved to isolate particles of various sizes (0.125-0.250 mm) for this work.

LEACHING OF COAL--CHEMICAL STUDIES

Reports on the leaching of organic compounds from coal appear to be non-existent,[93,98] although it is known that inorganic materials may be removed from coal storage piles by this process.[96-98]

Lakewater Leachate--

The leachate was prepared by stirring 11.1 g of coal (<0.125 mm) per liter of Lake Superior water for 48 hr. It was then centrifuged in a Sorvall (SS-3) continuous-flow centrifuge and 14.23 ℓ of the centrifugate was forced through a GF/F filter and a 50x7 mm column of C-18 Corasil as described in the analytical section (Section 5). Column-coupled reverse-phase HPLC produced a curve which is compared in Figure 1 with a similar curve obtained from a 14.48 ℓ blank of Lake Superior water. The water chemistry analyses are presented in Table 1.

Purified Water Leachate (Filtered)--

This leachate was prepared by stirring 10 g of coal (<0.125 mm) per liter of purified water (see Section 5 for preparation) for 48 hr. It was then placed into a stainless steel pressure can and forced through a Whatman GF/F filter which was replaced seven times in order to maintain a good flow. The filtered leachate was then forced through a GF/F filter-pre-column combination (see Section 5) and analyzed by column-coupled HPLC in the usual manner

(Section 5). The results are presented in Figure 2 and the water chemistry analyses are presented in Table 1.

Purified Water Leachate (Centrifuged)--

This leachate was prepared by stirring 10 g of coal (<0.125 mm) per liter of purified water (see Section 5 for preparation) for 48 hr. It was then run through a continuous flow centrifuge followed by the usual GF/F--pre-column--HPLC procedure (Section 5). The results are presented in Figure 3 and the water chemistry analyses are presented in Table 1.

Discussion

The results indicate that aqueous leachates of coal prepared as described at ambient temperature contain less than ∿10-50 ng/ℓ, if any, of individual polynuclear aromatic hydrocarbons. No PAH compounds were identified. The results presented in Figures 2 and 3 for purified water leachates, in contrast to the results in Figure 1 for lake water leachates, indicate the presence of significant amounts of polar materials (phenols?) not present in the blanks. Analysis of these polar compounds was not attempted.

VOLATILIZATION FROM COAL--CHEMICAL STUDIES

An open storage coal facility, such as the one in Superior, Wisconsin, which contains a large amount of high-moisture content coal that is periodically sprinkled with water to reduce dust liberation, has a high potential for steam volatilization of organic compounds. In this process, relatively low molecular weight compounds associated with coal may be volatilized at temperatures less than 100°C.[124] It should be noted that temperatures of 40-80°C above ambient temperature have been recorded at a Yorkshire coal pile and a number of alkanes and benzenes were found in nearby air.[125]

Experimental Details

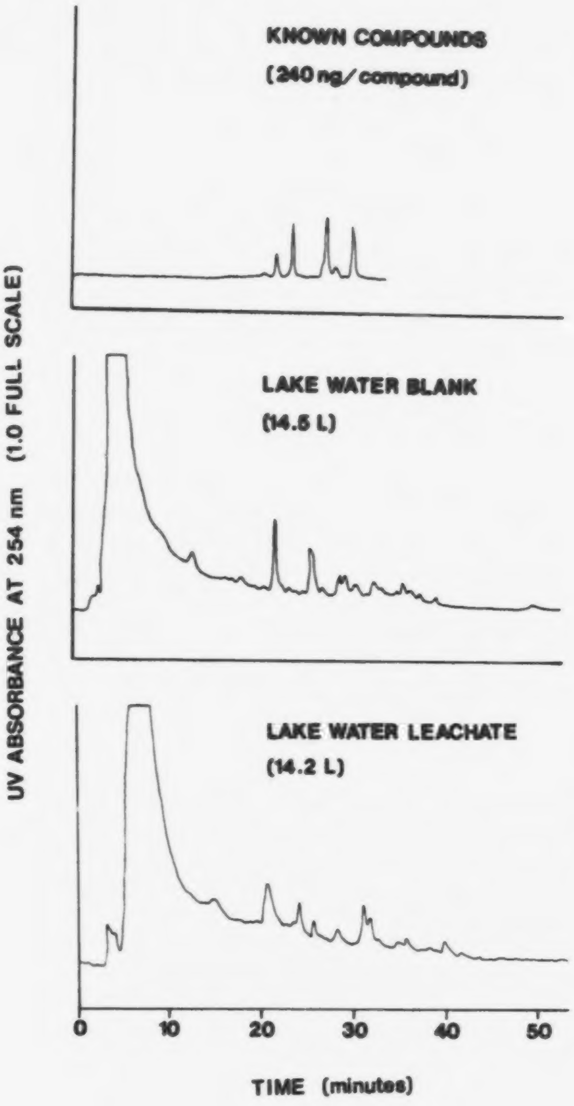
One hundred grams of coal (<0.125 mm) and 1.5 ℓ of purified water (see Section 5) were placed in a distillation apparatus. Additional water was added during the distillation process until 4 ℓ of distillate was obtained. In the second of two experiments, 4 g of KOH was added to the distillation flask in order to minimize the amount of sulfur and organic acids in the distillate. The two distillates were analyzed in the usual manner using the pre-column--HPLC--GC/MS procedure (see Section 5). The HPLC traces obtained are shown in Figures 4 and 5 and a list of compounds identified on the basis of their mass spectrum is provided in Table 2.

Discussion

The majority of the products volatilized from the coal by steam distillation appear to be alkanes, alkylbenzenes, and alkylnaphthalenes. The lack of unsubstituted PAH's is consistent with the high temperature requirement for the formation of these compounds.[126]

FIGURE 1

LAKE WATER LEACHATE LIQUID CHROMATOGRAMS



Conditions-column: 5μ Lichrosorb RP-18, 4.6 x 250 mm; program: 50-90% CH_3CN in water, 1.2 ml/min, 30 min (linear program). Compounds in standard-21.86 min: dibenzofuran; 23.23: fluorene; 24.92: phenanthrene; 28.67: 1-methylphenanthrene, fluoranthene; 29.73: pyrene; 31.56: 9-chlorophenanthrene.

TABLE 1

WATER CHEMISTRY ANALYSES FOR LEACHATES

	LAKE WATER	LAKE WATER LEACHATE[a]	PURIFIED WATER	PURIFIED WATER LEACHATE
Alkalinity (mg/ℓ $CaCO_3$)	42.49	7.62	1.04	4.12[a], 3.08[b]
EDTH Hardness (mg/ℓ $CaCO_3$)	43.84	2.90	0	0.0[a], 0.49[b]
pH (at 20^{0}C)	7.8	8.1	6.3	6.8[a],6.5[b]
Conductivity (μmhos/cm)	88	108	4.9	83[a], 87[b]
Turbidity	0.6	3.6	0.20	0.70[a], 2.25[b]

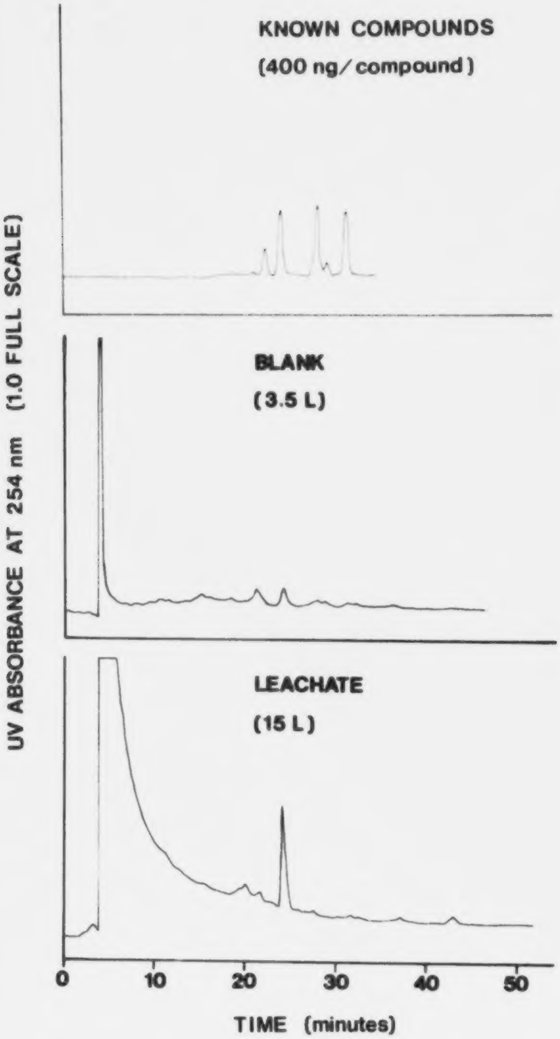
[a] Leachate prepared using a coal to water ratio of 10 g/ℓ, followed by 48 hr stirring and filtration through GF-F filters.

[b] Similar to (a) but the leachate was centrifuged.

FIGURE 2

PURIFIED WATER LEACHATE (FILTERED)

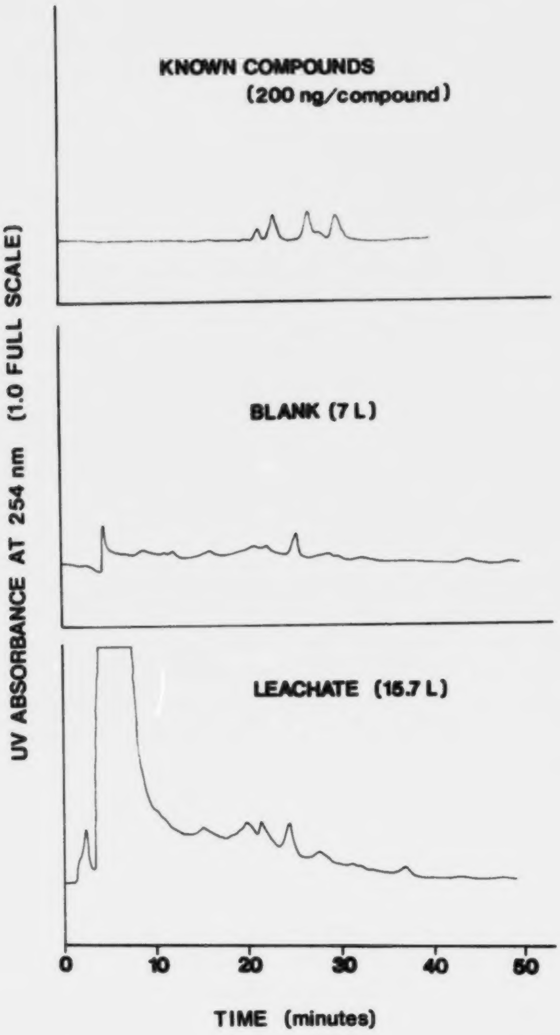
LIQUID CHROMATOGRAMS



Conditions-column: 5μ Lichrosorb RP-18, 4.6 x 250 mm; program: 50-90% CH_3CN in water, 1.2 ml/min, 30 min (linear program). Compounds in standard-19.85 min: dibenzofuran; 21.22: fluorene; 22.91: phenanthrene; 26.76: 1-methylphenanthrene, fluoranthene; 27.79: pyrene; 29.72: 9-chlorophenanthrene. Peak at 24.32 min in leachate experiment was shown to be phthalate ester contamination.

FIGURE 3

PURIFIED WATER LEACHATE (CENTRIFUGED) LIQUID CHROMATOGRAMS



Conditions-column: 5μ Lichrosorb RP-18, 4.6 x 250 mm; program: 50-90% CH_3CN in water, 1.2 ml/min, 30 min (linear program). Compounds in standard- 20.23 min: dibenzofuran; 21.61: fluorene; 23.31: phenanthrene; 27.20: 1-methylphenanthrene, fluoranthene; 28.28: pyrene; 30.19: 9-chlorophenanthrene. Peak at 37.38 min in leachate was analyzed by GC/MS but nothing was found.

LEACHING AND VOLATILIZATION OF COAL - BIOLOGICAL STUDIES

Biological testing of complex mixtures derived from fossil fuels has generally involved the study of acute and/or chronic toxicity and the investigation of sublethal effects (Table 3 and 4 and references cited therein). Using this same approach, leachates and steam distillates of Northern Great Plains coal were assessed for toxic effects on *Daphnia pulicaria* and fathead minnows (*Pimephales promelas*) and sublethal effects {growth, spawning behavior, cough response, bioaccumulation, and mixed-function oxidase (MFO) induction} using fathead minnows, sunfish (*Lepomis macrochirus*), and rainbow trout (*Salmo gairdneri*).

Experimental Details and Results

Preparation of Coal Leachate--

Eighteen liter batches of coal leachate were prepared by stirring 6.3 g of coal (<0.250 mm)/ℓ of distilled deionized or Lake Superior water for 72-96 hr. at ∿ 20°C. The leachates were usually clarified by continuous-flow centrifugation (Sorvall SS-3) at 12,000 rpm prior to use in the biological tests. Various properties of the leachate including dissolved oxygen, alkalinity, hardness, pH, conductivity, and turbidity are recorded in Table A-1 and Figure A-1. The gas chromatograms of methylene chloride and hexane extracts of centrifuged leachates are presented in Figures A-2 and A-3.

Preparation of Steam Distillate of Coal--

Coal distillates were prepared for fish experiments by boiling 2.5 of Lake Superior water with 100 g of coal (<0.25 mm) in a distillation apparatus and collecting 2 ℓ of distillate. For *Daphnia* experiments the amount of coal was increased to 625 g and distilled deionized water was used. The pH of the distillates varied between 5.0 and 6.8. The gas chromatogram of an isooctane extract of a distillate is presented in Figure A-4 and ultraviolet spectra of a distillate is presented in Figure A-5.

Toxicity Studies--

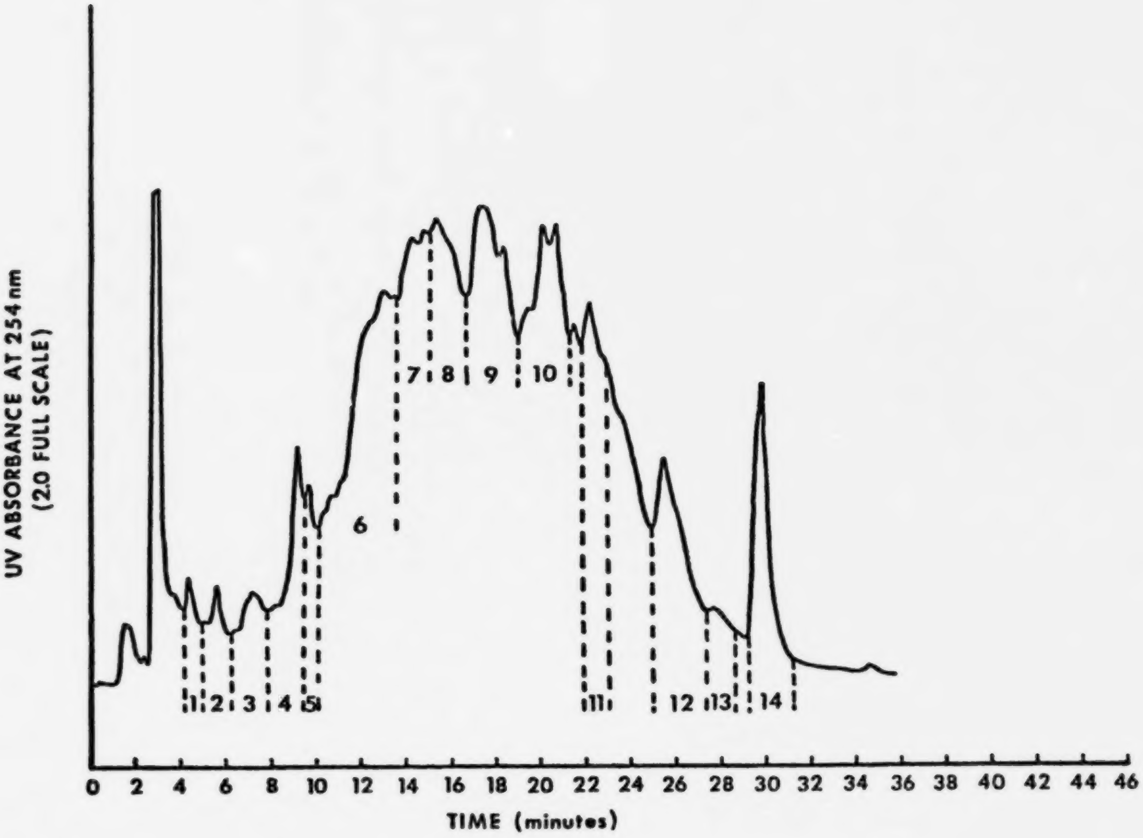
Coal leachates and distillates were tested for toxicity by exposing fathead minnows (*Pimephales promelas*) in static or renewed static bioassays. Exposure tanks were 10 ℓ glass aquaria containing 6 ℓ of test solution. Temperatures were maintained at 25±1°C and the photoperiod set at 16:8 (light:dark) in all experiments. The test water was aerated and renewed at 72-96 hr intervals during experiments exceeding 96 hr. Air was passed through a column of glass wool and charcoal before entering the tanks. In tests longer than 1 week, adults were fed frozen brine shrimp (San Francisco Bay Brand) and # 2 pellets (Zeigler Bros., Inc., Gardners, PA) and juveniles fed live brine shrimp nauplii at 2-5% of their body weight per day. Newly hatched larvae were exposed in glass jars (12 x 5 cm) with nylon-screened bottoms which were suspended in an exposure tank.

Coal distillate was also tested for toxicity to *Daphnia pulicaria* according to the method of Biesinger,[132] In each experiment five 24-hr -old

FIGURE 4

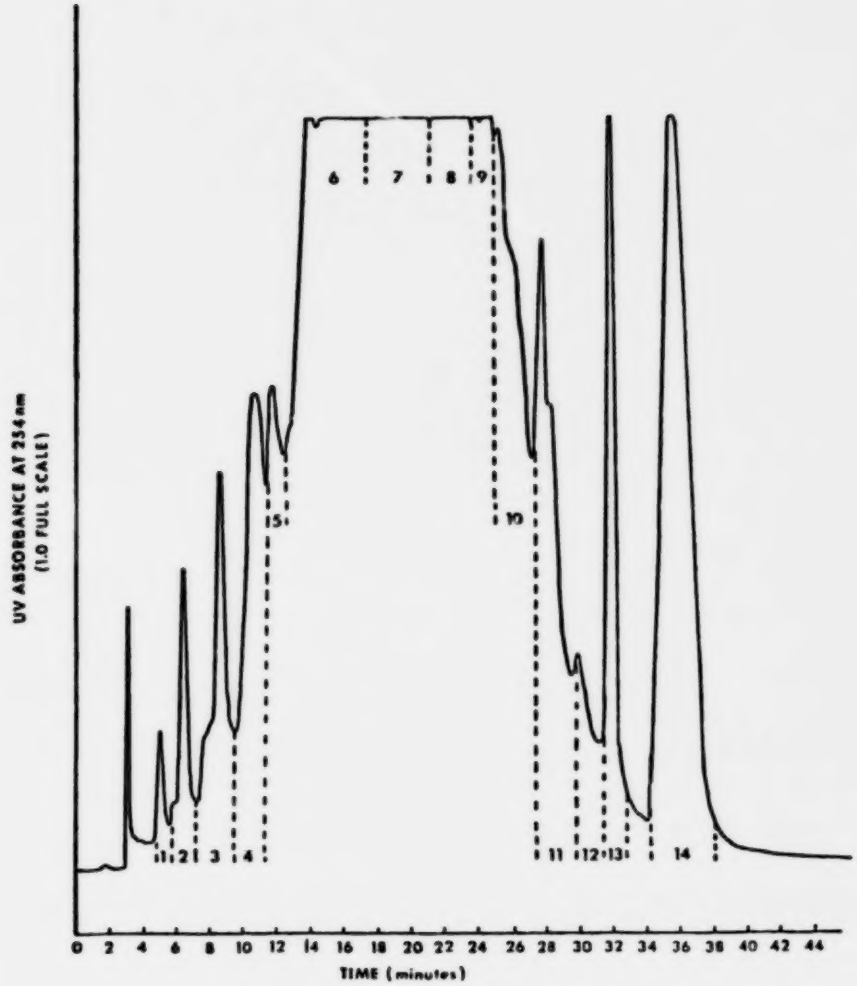
STEAM DISILLATE OF COAL (KOH)

HPLC



Conditions- column: 10 µ Lichrosorb RP-18; program: 50 to 90% CH_3CN in water, 1.2 ml/min, 30 min (linear program).

FIGURE 5
STEAM DISTILLATE OF COAL (NO KOH)
HPLC



Conditions- column: 10 µ Lichrosorb RP-18; program: 50 to 90% CH_3CN in water, 1.2 ml/min, 30 min (linear program).

TABLE 2

STEAM DISTILLATES OF COAL

RESULTS OF GC/MS ANALYSIS

			HPLC Fraction No.		Mass Spectrum No.	
Compound	m^+	Other Ions	Distillate with KOH	Distillate without KOH	Distillate[a] with KOH	Distillate[b] without KOH
alkanes, alkylbenzenes			F6, F7, F8			
c_2-biphenyl	182	167	F9		332	
c_2 naphthalenes	156	141	F9		260	
	156	141	F9		272	
	156	141	F9		282	
	156	141	F9		287	
	156	141	F9		293	
	156	141	F9		309	
c_3 naphthalenes	170	155		F8		117
	170	155		F8		120
	170	155		F8		125
	170	155		F8		132
	170	155	F-10		283	
	170	155	F-10		293	
	170	155	F-10		299	
	170	155	F-10		303	
	170	155	F-10		309	
	170	155	F-10		314	
	170	155	F-10		322	
	170	155	F-10		331	
c_4-naphthalenes	184	169		F9, 10		136
	184	169		F9, 10		141
c_5-benzene	148	133,105,91	F-10		209	
n-dodecane	170	85,71	F-10		212	
cyclododecane	168	97,83,69	F-10		230	
c_9-benzene	204	189,147	F-10		250	
c_2-hexahydrophenanthrene?	212	197	F-10		341	
methylphenanthrene/anthracene	192	191	F-10		418	
	192	191	F-10		431	
methylfluoranthene/pyrene	216	201		F-13		169
S_8	256	224,192,160 128,96		F-14		182

[a] GC conditions--column: 2 mm x 12' with 5% SP-2250 on 100/120 Gas Chrom Q; program: 80-225°C @ 2°/min. Mass spec: start scan after 2 min; scan: 50 to 300 amu @ 7.5 sec/decade.

[b] GC conditions--column: 1/8" x 6' with 3% OV-101 on 80/100 Gas Chrom Q; program: initial hold 1 min, 80 to 225°C @ 4°/min. Mass spec: start scan after 2 min; scan: 50 to 300 amu @ 7.5 sec/decade.

TABLE 3

REPORTED STUDIES ON THE BIOLOGICAL EFFECTS OF THE WATER SOLUBLE FRACTION OF PETROLEUM ON FISH

Type of Test	Organism(s)	Effect	Reference
Toxicity, Acute	Salmon, trout, cod	24, 96-hr toxicity	127
Sublethal Growth/ Development	Baltic herring	decreased lengths, malformed larvae	128
Cough Response	Salmon	increased cough rate	129
MFO	Brown trout, cunner, copelin	AHH introduction	130
Bioaccumulation	*Fundulus*	bioaccumulation of naphthalenes	46

TABLE 4

REPORTED STUDIES ON THE BIOLOGICAL EFFECTS OF COAL-DERIVED MIXTURES ON AQUATIC ORGANISMS

Test Solution	Type of Test	Organism	Effect	Reference
fly ash leachate	toxicity (embryo larval)	goldfish redear sunfish	Mortality (24-72 hr)	131
coal conversion gasifier condensate	toxicity	fathead minnow	Mortality (24,48,96 hr)	112
resorcinol + 6-methyl quinoline	toxicity	*Daphnia*	Mortality (48 hr)	37

daphnids were placed into a 250-$m\ell$ beaker containing 200 $m\ell$ of solution of 20, 40, 60 or 80% coal distillate in Lake Superior water. Controls were prepared using the corresponding amount of distilled water in Lake Superior water. The temperature was 18±1°C and the photoperiod was 16:8 (light:dark).

The results (Table 5) indicate no toxic effects of centrifuged leachate to juvenile or adult fathead minnows. However, uncentrifuged leachate caused 25% mortality during the first 2 weeks of a 24-week exposure (6.3 g or coal/ℓ) and 100% mortality during a 96 hr bioassay (25 g coal/ℓ).

Coal distillate caused no significant mortality to adult or newly-hatched fathead minnows (Table 6) at the highest concentration tested (20%). Partial mortality of *Daphnia* exposed to 20, 40 and 60% coal distillate was noted in 1 out of 3 bioassays (Table A-2). The consistent mortality observed at 80% levels was also found at comparable distilled water levels, indicating that the low ionic strength of the test water was the lethal factor.

Sublethal Effect Studies--

Growth--The growth rates of two-month-old fathead minnows exposed to coal leachate (distilled deionized water) were compared to fish exposed to purified Lake Superior water (XAD-2) in a 24-week experiment.* The leachates for the first two weeks of the experiment were not centrifuged and a 50% mortality of fish was noted in one leachate tank. Thereafter, centrifuged leachate was used for the experiment. Exposure water was renewed twice a week and the 6 ℓ tanks were continuously aerated. Temperatures were maintained at 24±1°C and the photoperiod set at 16:8 (light:dark). The fish were 2 months old at the onset of the experiment and the maximum loading attained at the termination of the experiment was 2.7 g fish biomass per liter of tank water. The fish were fed 5% of their body water per day in 4 feedings. Food consumption was measured weekly, while total fish weights were determined every two weeks. Daily observations were made for signs of male spawning coloration. Growth curves and maturation data are presented in Figure A-7 and Table A-3 (Appendix A) and indicate a similar growth rate, but an apparent retardation in maturation.

Spawning--The spawning behavior of fathead minnows exposed to coal leachate (made using Lake Superior water) was compared to fish exposed to only Lake Superior water in a number of 2-4 week experiments. In each experiment 2 male and 4 female fathead minnows (9 mo - 1 yr) were exposed to either water or leachate in a tank containing three spawning substrates prepared by gluing (Corning black silicone) a layer of sand to the inside of glass half-cylinders (60cm radius). Eggs were removed and counted daily. Hatchability in Lake Superior water was determined by incubating groups of 50 eggs in glass jars which were mechanically oscillated. In these experiments the fish were fed frozen brine shrimp and pellets twice a day. The

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* Gas chromotograms of centrifuged leachate and purified Lake Superior water are shown in Figures A-2, A-3 and A-6

TABLE 5

SUMMARY OF BIOLOGICAL TESTING
OF COAL LEACHATE.[a]

Concentration	Organism	Age at Onset	Duration of Exp.	Type of Test	Bioassay Conditons	Response
100% (6.3g/l DDW)	fathead minnow	5-15 days	3 weeks	Toxicity	Renewed Static	15% mortality not significantly greater than controls
100% (6.3g/l DDW)	fathead	2½ mo	24 weeks	Toxicity	Renewed Static	No mortality with centrifuged leachate
100% Uncentrifuged (25g/l DDW)	fathead minnow	2½ mo	96 hours	Toxicity	Static	100% mortality
100% (6.3g/l DDW)	fathead minnow	2 mo	24 weeks	Sublethal Growth Bioaccumulation	Renewed Static	Growth rate similar to that in Lake Superior water but onset of maturity delayed. Some qualitative differences in GC analyses of tissue extracts
10-100% (6.3g/l LW)	fathead minnow	~1 yr	2 or 4 weeks	Sublethal: Spawning	Renewed Static	36% spawning success in leachate exposures. 90% spawning success in control exposures.
100% (6.3g/l LW)	rainbow trout	1 yr	28 days	Sublethal: AHH Response Liver Parameters	Renewed Static	No consistent differences from controls

[a] The ratio of coal to water and the type of water used to prepare leachates (100%) are indicated. DDW = distilled deionized water, LW = Lake Superior Water

TABLE 6
SUMMARY OF BIOLOGICAL TESTING OF COAL DISTILLATE.[a]

Concentration	Organism	Age at Onset	Duration of Exp.	Type of Test	Bioassay Conditions	Response
20, 40, 60%	*Daphnia*	≤24 hr	48 hr	Toxicity	static	Death at high concentrations due to low conductivity. Partial mortality at intermediate concentrations.
20% (67g/l, DDW)	fathead minnow	24-48 hr	96 hr	Toxicity	static	No different from controls
0.1,1,10,20% (67g/l, DDW)	fathead minnow	9 mo	96 hr	Toxicity	static	No mortality
1,5,10,20% (67g/l, DDW)	bluegill	?	24 hr	Sublethal: Cough Response	recirculating static	No sustained, elevated cough rate
0.2% (40g/l LW)	rainbow trout	1 yr	21 days	Sublethal: MFO Response Bioaccumulation	flow-through	P-450 no different from control Slight AHH elevation, D28 No different from control

[a] The ration of coal to water and the type of water used to prepare distillates (100%) are indicated. DDW= distilled deionized water, LW = Lake Superior Water.

tanks were aerated and the water renewed twice a week. Spawning success (Table A-4) is defined as the percentage of exposure tanks in which at least one spawning occurred during the 2-4 week experimental period. There was only a 36% spawning success in the 22 leachate exposure tanks compared to a 90% spawning success in 10 control exposure tanks. In spite of the overall lower spawning success observed during the leachate exposures, spawning ultimately occurred at all leachate concentrations tested.

Cough Response--Two bluegill sunfish (*Lepomis macrochirus*) were exposed to 1, 5, 10 and 20% concentrations of coal distillate in tanks designed to monitor opercular movement.[133] Exposure tanks contained 4 ℓ of water, and fish were exposed under recirculating static conditions. Physiographs were activated 1 hr before introduction of distillate to obtain baseline data for each fish. After the test was initiated, 20-min scans were picked at selected intervals and the frequency of the cough response tabulated. The results of this experiment are presented in Table A-5. In both tanks the initial elevation of cough rate rapidly diminished indicating a lack of imminent toxicity.

Hepatic Mixed-function Oxidase Activity (coal leachate)--Rainbow trout (1 yr old, 10-15 cm) were exposed to 100% coal leachate for 28 days in order to determine the effect on hepatic mixed-function oxidase activity and other liver parameters. These fish, which had been held in flowing Lake Superior water for three months prior to use, were exposed in 40 ℓ glass aquaria containing 100% leachate (made with Lake Superior water) or Lake Superior water at 11±1°C under subdued lighting (16:8). The water in each tank was renewed every 72 hr and vigorous aeration maintained. The mean conductivity of the water control tank was 91 μmhos/cm. The fish were fed 1% of their body weight in two daily feedings. Samples consisting of three fish were removed from each tank on days 1, 3, 7, 21 and 28. Microsomes, prepared from the livers of these fish, were analyzed for aryl hydrocarbon hydroxylase activity (AHH) by methods described in the mixed-function oxidase discussion (Section 6). Hepatic DNA context, microsomal protein, and relative liver weight were also assayed at each sampling period. The DNA content of the 15,000 g pellet from the homogenized liver preparations was analyzed according to Burton.[134] Fifteen-minute extractions of the pellet were done at 70° using 3 mℓ 0.5 N perchloric acid. During the first extraction, 2 drops of 0.5% sodium dodecyl sulfate were added in addition to the acid. After adjusting the total volume to 10 mℓ with perchloric acid, 200 μℓ was then assayed for DNA concentration. The incubation mixture contained 200 μℓ DNA extract, 500 μℓ 0.5 N perchloric acid, and 2 mℓ freshly prepared diphenylamine reagent. Duplicate tubes plus DNA standards and a reagent blank were all incubated at 30°C for 10-20 hr and then read against the blank at 600 nm on a Beckman DB-G spectrophotometer. Calf thymus DNA (Sigma) was used to prepare standards.

Protein was determined by the biuret method.[135] Five-hundred μℓ samples of microsomes were combined with 2 mℓ of freshly prepared biuret reagent and the absorbance at 550 nm was determined. Crystalline bovine serum albumen was used as the standard.

Relative liver weight was determined by dividing the wet weight of each liver by the corresponding total fish weight. The values shown in Figure A-8 represent the mean ratios from three fish.

Leachate exposure resulted in no consistent liver changes during the 21-day experiment (Figure A-8). Hepatic DNA content, microsomal protein and relative liver weight in leachate-exposed fish were similar to values obtained during lakewater exposure. Although a significant elevation of AHH activity was noted on day 7 of the experiment, no elevated activity was maintained.

Hepatic Mixed-function Oxidase Activity (coal distillate)--In a related experiment, rainbow trout were exposed to 0.2% coal distillate for 21 days under flow-through conditions. Distillate was metered into a 55 ℓ stainless steel tank using an FMI (Fluid Metering, Inc.) lab pump with stainless steel fittings and tubing. Flow rates were 27.5 ℓ/hr for the distillate tank and 40 ℓ/hr for the lake water (control) tank. The trout were kept at 11±1°C under subdued lighting (16:8). Samples of three fish each were removed on days 3,7,10,14 and 21. The livers were pooled and assayed for cytochrome P-450 concentration and AHH activity by methods described in the mixed-function oxidase discussion (Section 6). The results of this experiment are presented in Table A-6 and indicate an overall lack of MFO induction. There was, however, a small but observable elevation in AHH activity on day 21 of the experiment.

Bioaccumulation (coal leachate)--In order to determine if there were any qualitative differences in bioaccumulation between the fish exposed to coal leachate and the control fish in the 24-week growth experiment described previously, extractions from the fish were analyzed by gas chromatography. Fish from each tank were combined and frozen until analysis. The fish were ground in a blender with 40 g solvent-washed sodium sulfate and Soxhlet extracted with methylene chloride for 4 hr. The extracts were evaporated under a hood, transferred to 15 mℓ centrifuge tubes and then evaporated to exactly 3 mℓ. Interfering lipids were removed by gel permeation chromatography[136] with redistilled, deaerated methylene chloride as the carrier solvent. After concentrating in a Kuderna-Danish evaporator, the sample was injected onto a gas chromatograph (Figure A-9). Results indicate qualitative and quantitative differences in bioaccumulated material.

Bioaccumulation (coal distillate)--In order to determine if there were any qualitative differences in bioaccumulation between fish exposed to coal distillate and control fish in the 21-day hepatic mixed-function oxidase experiment described previously, the gutted fish were extracted and analyzed by gas chromatography. The results are presented in Figure A-10 and showed no major differences in the chromatograms.

Discussion

Biological testing of material liberated from Northern Great Plains coal indicated no severe adverse effects on fathead minnows, rainbow trout or *Daphnia*. Toxicity, growth retardation and MFO induction were not observed in fish exposed to coal leachate, although a lowered spawning success rate was noted in fathead minnows. Distillate effects were limited to slight *Daphnia* mortality at intermediate concentrations (20, 40, 60%) and a small increase in hepatic MFO activity in rainbow trout.

However, conclusive statements cannot be made about the impact of leached and volatilized materials on an actual receiving water due to the difficulty in defining what would constitute a natural exposure setting. Moreover, toxicity can be ascribed to the presence of particulate matter (i.e. uncentrifuged leachate) and/or low conductivity of the water (i.e. high concentrations of distillate) rather than to the presence of specific toxic compounds.

SECTION 5

GENERAL ANALYTICAL TECHNIQUES DEVELOPED FOR PROJECT

This section describes the general analytical techniques that were developed during this project for the analysis of polynuclear aromatic hydrocarbons and their derivatives in water and fish tissue.

WATER ANALYSIS

The determination of PAH present in water at the microgram per liter level or less[137,138] has usually been carried out by removal of the PAH material from water using liquid-liquid extraction techniques[139-142], headspace sampling techniques[143-145], or adsorption techniques employing materials such as Tenax GC[146-148], XAD resins[92,149-151], polyurethane foam[152,153], or C-18 Corasil[143,144,154], followed by analysis of this material with high performance liquid chromatography (HPLC)[143,144,154], gas chromatography (GC)[140,142,147], and gas chromatography/mass spectrometry (GC/MS)[142,144]. Direct analysis with fluorescence has also been attempted[155].

Results and Discussion

For the present work it was decided to use an adsorption technique because liquid-liquid extraction techniques require the use of large volumes of organic solvents followed by a tedious and time-consuming concentration step, and extensive data on the successful application of headspace sampling techniques to analysis of PAH in water is lacking[145]. The adsorbent chosen was C-18 Corasil^{R} because a convenient procedure for analysis of the adsorbed material via C-18 pre-column-coupled reverse-phase HPLC has been reported[138,143,144]. However, reverse-phase HPLC alone does not give extensive separation of various PAH's for accurate identification and quantitation. Therefore, we have improved upon this procedure[156] by the introduction of several modifications, including the use of a gas chromatographic separation (photoionization detection).

The procedure begins with forcing an aqueous sample through a glass microfiber filter (necessary for environmental samples) connected in series with a 50 x 7 mm column packed with C-18 Corasil^{R}. The column containing the PAH material is then attached to a C-18 micro-packed reverse-phase HPLC set-up and eluted with an acetonitrile-water gradient. Fractions corresponding to each UV peak are collected and these aqueous-acetonitrile fractions are then injected directly into a GC equipped with a photoionization detector (PID). The procedure thus provides compound identification and quantitation data from both HPLC and GC and is very convenient since no concentration or water removal steps are required unless GC/MS analysis is necessary.

The results of the PAH recovery determinations (Table 7) indicate that sub-micron filters such as the Whatman GF/F (effective retention of 0.7 micron) which are often part of the analysis procedure have little effect on the recovery of PAH's from water. In contrast, the Whatman GF/B filter (effective retention of 1.0 micron) did lower the recovery of several PAH's in the one experiment in which it was used.

The HNU Systems photoionization GC detector[157] used in this work is at least ten to forty times more sensitive to PAH's (lower limit of detection ∿0.05 to 0.1 ng) than a flame ionization detector (FID) and, in contrast to the FID, shows little response to water or acetonitrile. The lack of a significant solvent peak allows the use of isothermal GC conditions for many experiments, which results in greater reproducibility of retention times and a more stable baseline. This detector, which has a maximum temperature limit of 250°C, gradually becomes fogged which results in a decrease in sensitivity. In order to regain maximum sensitivity it is necessary to remove the lamp for cleaning with an abrasive cleaner provided by the manufacturer. The linearity and reproducibility varied somewhat[157] as is reflected by the variations in the errors given in Table 7.

EXPERIMENTAL DETAILS

Apparatus--

Stainless steel pressure tanks (Amicon, Model RS20) with internal volume of 19.5 ℓ were pressurized up to 125 psi with cylinders of Linde high purity nitrogen which was pre-purified *via* an in-line 7 x 50 mm Porapak^{R} QS column. Stainless steel and brass were used for all fittings and lines.

Stainless steel filter holders (Gelman part number 2220, 47 diameter) were used to hold Whatman or Schleicher and Schuell (S&S) glass microfiber filters.

Stainless-steel HPLC columns (7 x 50 mm) were dry-packed with Bondapak C-18 Corasil^{R} II or Porasil^{R} B (37-50 μ) (Waters Associates).

The HPLC apparatus was manufactured by Waters Associates and consisted of the following items: two M-6000 pumps, a model 660 solvent programmer, a model U6K injector, a model 440 dual channel UV detector equipped to monitor 254 and 280 nm, and a 3.9 x 100 mm 10 μ C-18 reverse phase column (or a 4.6 x 250 mm 5 μ C-18 Lichrosorb column manufactured by Altex Associates).

Swagelok^{R} stainless steel quick-connect fittings for 1/16 inch tubing were used on the pressure tanks, the 7 x 50 mm HPLC columns, and the micro-packed analytical HPLC column.

The GC apparatus consisted of a Tracor 550 chromatograph equipped with an HNU Systems photoionization detector (model PI-51 max temp = 250°). The GC inlet was modified by welding a Waters Associates HPLC septum inlet onto the front of the Tracor septum nut in order to move the location of the septum (Hewlett-Packard, part number 5080-6721) about 3.5 cm away from the 300° inlet tube. The PID was connected to the Tracor outlet *via* a 1/16-inch

TABLE 7

DETERMINATION OF PAH RECOVERY FROM AQUEOUS SOLUTIONS OF KNOWN CONCENTRATIONS

USING THE C-18 ADSORPTION--HPLC-GC PROCEDURE

Experiment No.	1	2	3	4	5	6	7	8	9	10
Conc of PAH in ng/ℓ	80	80	80	22	80	80	107	600	1000	965
# of ℓ through C-18 column	14.5	14.3	14.2	18.2	14.0	12.2	13.8	6.4	6.2	10.2
# of μg of PAH for quantitative recovery	1.16	1.14	1.14	0.392	1.12	0.978	1.48	3.84	6.20	9.84
Glass fiber filter	None	a	b	b	c	c	c	c	None	None
Type C-18 packing	d	d	d	d	d	d	d	d	e	e
% Recovered:										
1-Methyl-naphthalene	--	--	--	--	--	--	--	--	--	76±10
Fluorene	76±3	64±6	77±11	42±8	72±18	70±20	69±4	93±16	89±13	--
Dibenzofuran	--	--	--	--	--	--	--	83±10	80±8	--
1-Methyl-4-chloro-naphthalene	--	--	--	--	--	--	--	--	--	95±2
Phenanthrene	87±3	101±2	87±2	105±21	85±2	90±4	--	--	--	--
2-Methylanthracene	81±24	64±5	72±11	115±18	61±9	87±13	74±2	--	--	--
1-Methylphenanthrene	87±10	78±13	82±13	99±68	87±22	111±12	86±10	--	--	--
9-Methylanthracene	103±21	92±13	87±24	--	101±17	110±18	106±12	--	--	--
Fluoranthene	96±18	101±16	87±20	81±5	67±4	76±9	88±14	--	--	--
Pyrene	65±16	84±28	79±35	92±55	36±10	100±11	106±14	--	--	--
9-Chlorophenanthrene	--	--	--	--	--	--	--	88±7	86±5	--

The errors given in this table reflect the standard deviation in the determination of the weight of material injected into the GC plus the error involved in the measurement of the volumes of the HPLC fractions.

a - S & S 29   b - S & S 30   c - Whatman GF/B   d - Waters Corasil II   e - Waters Porasil B stainless steel tube placed inside of a heated steel block. The purge inlet of the PID was also connected. Linde pre-purified grade nitrogen, which was run through a gas filter packed with Drierite^R and molecular sieves (Pierce Chemical Company, part number 06116.23), was used for the carrier and purge gases. Injections were made with a one-microliter Hamilton #7001 syringe fitted with a 7-cm needle. The glass GC column (8 ft x 20 mm ID x ¼" OD) was packed in the coil with 1.5% SP-2250/1.95% SP-2401 (methyl phenyl silicone and fluoropropyl silicone) on 100/120 Supelcoport^R (Supelco catalog number 1-1947; maximum temperature rated at 250^o) and in the ends with Anakrom flux-calcined diatomaceous earth (110/120 mesh; Analabs) in order to allow 300^o inlet and outlet temperatures with minimum column bleed. Supeltex^R M-2 column ferrules (Supelco) were used. The following GC conditions were typically employed: carrier flow rate = 23 ml/min, purge flow = 0, inlet temperature = 300^o, oven temperature = 182^o, outlet temperature = 305^o, auxiliary block temperature = 312^o, detector temperature = 250^o. On certain occasions increased reproducibility of detector response could be achieved, at some sacrifice in sensitivity, by adjusting the detector purge to ∿10-75 ml/min. An example of a typical gas chromatograph is provided in Figure 6.

Integrations of HPLC and GC peaks were carried out with a Hewlett-Packard 3380S integrator.

The GC-MS work was performed by electron impact at 70 eV on a Varian MAT CH-5 single focusing instrument equipped with Varian 620-I and 620-L data systems.

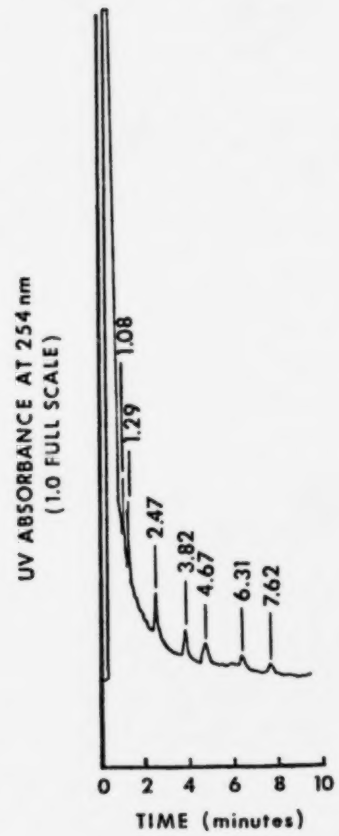
Distilled water was further purified for this work by forcing it through an 0.45 μ Millipore filter and a 7 x 600 mm Bondapak C-18 Porasil B reverse-phase HPLC column (Waters Associates).

Determination of PAH Recovery from Solutions of Known Concentration--

An aqueous solution of known PAH concentration was prepared by adding, with stirring, the appropriate amount of an acetonitrile solution of PAH to "purified" water contained in a 19-ℓ pressure tank *via* a 100-μℓ syringe. The filter holder assembly and the 7 x 50 mm C-18 column were then attached to the outlet and the pressure inside the tank was adjusted to maintain a flow rate of about 20-30 ml/min. If necessary, the column was stored at 5^{o}C prior to HPLC-GC analysis. The column was then inserted into the HPLC system directly in front of the reverse-phase column and the following linear solvent program was run: 1:1 acetonitrile-water to 9:1 acetonitrile-water over a 30-minute period with a total flow rate of 1.2 ml/min. Fractions corresponding to various peaks were collected in glass-stoppered, graduated 5-ml centrifuge tubes. A one-microliter sample from each tube was then immediately injected into the GC along with 3-8 injections of PAH solutions of various known concentrations. A computer program was used to calculate a least squares line (GC peak height or area versus weight injected) for each PAH in the solutions of known concentration and to calculate from this line the amount of each PAH that occurred in the HPLC fractions. The results are tabulated in Table 7. The errors given in this table reflect the standard deviation in the determination of weight of material injected[158], plus the

FIGURE 6

TYPICAL GAS CHROMATOGRAM OF PAH MIXTURE USING PHOTOIONIZATION DETECTOR



Injection: 1 µl of a 0.2 ng/µl acetonitrile solution of dibenzofuran (1.08), fluorene (1.29), phenanthrene (2.47), 1-methylphenanthrene (3.82), 9-chlorophenanthrene (4.67), fluoranthene (6.31), and pyrene (7.62).

error involved in measurements of the volumes of the HPLC fractions. (See Appendix B).

Analysis of Samples of Unknown Composition--

For analysis of environmental samples, coal leachate samples and other unknown solutions, the filter holder was fitted with a Whatman GF/F filter and the analysis proceeded as described above. The filter was replaced as often as necessary to maintain the flow rate of 15-30 *mℓ*/min. For GC-MS identification work it was necessary to remove the water from the individual HPLC fractions and to concentrate them. The water removal was effected by addition of ∿0.5 *mℓ* of methylene chloride to cause separation of layers, followed by removal of the aqueous layer with a Pasteur pipette. The organic layer which remained was dried with sodium sulfate and concentrated under a stream of nitrogen.

FISH ANALYSIS

The analysis of PAH in biological tissues usually involves removal of the PAH by extraction[145,159], alkaline digestion/extraction[145,159], or headspace sampling[145], followed by clean-up *via* column chromatography on alumina or silica[159], reverse-phase or adsorption HPLC[145], or gel-permeation chromatography[160] with subsequent analysis by GC, GC/MS, ultraviolet and/or fluorescence.

Results

For the present work a procedure was developed which employs an extraction-HPLC/Styragel^{R}-GC/PID and/or GC/MS sequence. The Styragel^{R} step (size separation) provided excellent separation of PAH material from biomolecules. The resulting PAH fractions were analyzed directly (no concentration required) *via* gas chromatography with photoionization detection or concentrated for GC/MS analysis as described above. The results of determinations of recovery efficiencies for the procedure with fish samples spiked with known amount of PAH prior to the extraction step are provided in Table 8.

Experimental Details

Apparatus--

The HPLC apparatus was manufactured by Waters Associates and consisted of the following items: an M-6000A pump, U6K injector, 254 nm UV detector and two 7.8 x 1220 mm 60A Styragel columns connected in series. Methylene chloride was used at a flow rate of 4.0 *mℓ*/min. An example of a typical HPLC fractionation is provided in Figure 7. The gas chromatograph used for this work was described above.

Procedure--

The fish (∿2-5 grams) and ∿15 g of sodium sulfate were ground in a Waring blender. The material obtained was mixed with an additional 15 g of sodium sulfate and extracted in a Soxhlet extractor with 170 *mℓ* of methylene chlo-

TABLE 8

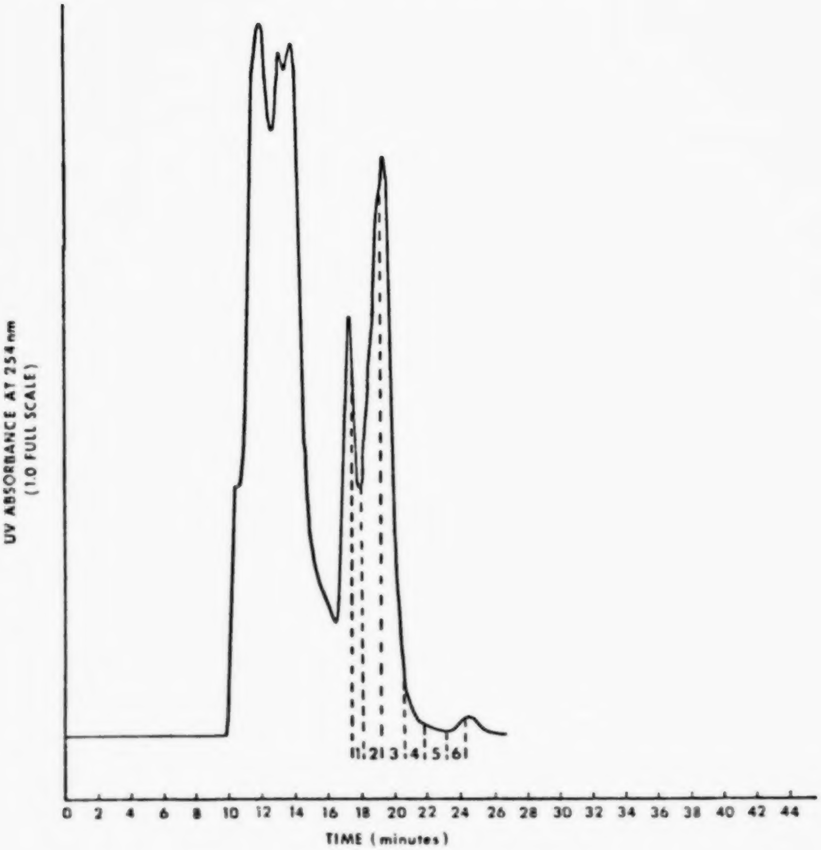
SUMMARY OF ANALYSES OF SPIKED FISH TISSUE SAMPLES

Trial #	Grams Fish Added	µg PAH Added	mg PAH / kg fish	% Recovered β-Naphthoflavone	Dibenzufuran	Fluorene	Phenanthrene	1-Methyl-phenanthrene	9-Chloro-phenanthrene	Fluoranthene	Pyrene
1	4.3	20.0	4.7	---	101±12	95±12	---	---	91±10	---	---
2	4.13	5.0	1.2	---	64±6	72±10	---	---	57±6	---	---
3	5.5	5.0	0.9	---	86±18	83±13	---	---	---	---	---
4	5.8	5.0	0.9	---	76±6	77±5	--	---	101±15	---	---
5	5.9	10.0	1.7	---	85±5	99±4	---	---	66±9	---	---
6	4.1	5.0	1.2	---	---	---	75±2	103±12	---	---	---
7	3.1	5.0	1.6	---	---	---	67±2	102±7	---	---	---
8	6.0	5.0	0.8	135±20[a]	---	---	---	84±12	---	---	---
9	7.4	10.0	1.4	84±7[a]	---	---	106±8	107±8	---	---	---
10	4.6	15.0	3.3	---	---	---	111±9	112±9	---	---	---
11	4.1	30.0	-7.3	83±3[a]	---	---	116±9	118±7	---	---	---
12	2.6	5.0	1.9	---	94±18	---	105±10	---	---	100±5	---
13	3.4	10.0	2.9	---	94±13	75±18	90±2	81±2	---	96±6	103±11
14	3.8	20.0	5.3	---	92±9	76±9	---	88±6	---	79±5	90±1
15	3.3	40.0	12.0	---	72±10	57±5	109±12	80±4	---	---	---

[a] Determined by HPLC-UV. All other determinations by GC-PID.

ride for 5 hours. The extract was then concentrated in a Kuderna-Danish apparatus to ∿1.5 *mℓ*. The concentrate was injected into the HPLC and fractions containing the PAH material were collected at ∿18-25 min (flow rate = 4 *mℓ*/min). One-microliter samples of each of the HPLC fractions were injected into the GC/PID along with injections of solutions of known concentration for quantitation as described in the water analysis section.

FIGURE 7
TYPICAL FRACTIONATION OF FISH TISSUE EXTRACT CONTAINING PAH MATERIAL USING HPLC-STYRAGEL



Sample: Methylene chloride extract of twelve fathead minnows (1.2738 gram total). Compounds: fluorene (fractions 1,2; 3.6 mg/kg fish), dibenzofuran (fractions 2,3; 3.8 mg/fish), phenanthrene (fractions 2,3; 4.9 mg/kg fish), 1-methylphenanthrene (fractions 2,3; 1.7 mg/kg fish), fluoranthene (fractions 2,3; 2.6 mg/kg fish), pyrene (fraction 3; 0.69 mg/kg fish).

SECTION 6

ENVIRONMENTAL IMPLICATIONS OF DISSOLVED POLYNUCLEAR AROMATIC HYDROCARBONS

UBIQUITY OF POLYCYCLIC AROMATIC HYDROCARBONS

The significance of the chemical and biological studies on specific PAH's extends well beyond the present discussion because these compounds and other PAH's can be derived from many sources and are considered ubiquitous.

The origins of polynuclear aromatic hydrocarbons found in the environment[101,126,161,162] are: a) synthesis by microorganisms[101,161] and plants [101,161,162]; b) geochemical processes[126] which result in the formation of coal[163] and petroleum; c) natural pyrolytic processes such as forest fires [126], prairie fires[126], and volcanoes[164]; and d) man-produced pyrolytic processes[162] such as coal-fired electrical generating plants. The latter man-produced origins are considered to be the most significant[101]. The pyrolytic formation of PAH's from organic matter involves free radical reactions[165-167] and possibly "benzyne" intermediates[167]. Higher temperatures generally result in formation of less alkylated PAH's[126].

The environmental cycle of PAH's has been discussed in several reviews [101,168-170] and a summary has been provided by Suess[101]:

> "The transport pattern of PAH in the environment appears to be relatively simple. The background PAH, which are formed by biosynthesis, are quite static and, obviously, remain in the plants and microorganisms in which they were formed and, more generally seen, stay within their own ecosystems be it the soil, which holds the synthesizing bacteria and the plant roots, or the lake, river or sea with its aquatic biota. However, it appears probable that PAH in ground-water are leached out from the soil.
>
> In contrast, PAH formed by high temperature processes, whether resulting from natural open burning and volcanic eruptions or from man-induced combustion reactions including ground, sea and air transportation, are all emitted into the atmosphere, and thus are subject to the same dynamic forces which govern the movement, transport and fallout of aerosols generally. Because a significant portion of PAH, absorbed onto the aerosols, will decompose by photooxidation while still in the atmosphere, either stationary or in motion, their fallout at great distances from

> the source (delayed fallout), will be relatively very limited. However, where fallout of PAH occurs, it will contaminate the upper layers of the earth, including vegetation and forests, as well as rivers, reservoirs and lakes, and some of them will also reach the oceans. Runoff and the rivers will carry eventually some of this fallout to the open seas and oceans. As waste treatment plants do not remove all PAH, the coastal waters will receive an additional load from domestic and industrial waste effluents either directly, or indirectly through the rivers. Such effluents will also carry PAH coming with oil pollution. Some of the PAH, settling on land and vegetation, are bound to be washed into the soil with minor amounts eventually reaching ground-water. The open seas and oceans will also be polluted by PAH from activities connected with oil transport of tankers, including loading and unloading, as well as oil spills and accidents at seas."

Polycyclic aromatic hydrocarbons in aquatic systems may be in the dissolved state[168,177], associated with particulate matter[161,168,169,171] and/or associated with organic matter[161,171]. Photooxidation[101,161,172,173], is probably one of the principal transformation processes that occurs in water and atmospheric environments. Particulates may enhance the rate of reaction[173-175]. Biological transformation processes in soil and sediment are also important, especially for smaller ring PAH's[176].

The levels of PAH's in the environment have also been discussed in several reviews[101,161,162,168,170,177,178]. The results of some recently published work on the identification and quantitation of PAH in the environment (tabulated in Table 9) indicate that although levels may vary considerably, PAH material may be found almost anywhere. It has been demonstrated that industrialization has elevated the levels considerably[101,102,162].

The present studies that will be discussed are a) the induction of mixed-function oxidases (MFO) in fish by specific PAH's, b) the bioaccumulation of PAH in fish and the possible relation between bioaccumulation and MFO induction, and c) the identification and quantitation of products of aqueous chlorination reactions of PAH.

TABLE 9

IDENTIFICATION AND QUANTITATION OF POLYNUCLEAR AROMATIC HYDROCARBONS: RESULTS FROM SELECTED LITERATURE REPORTS

Source	Compounds	$\mu g/10^3 m^3$	$\mu g/kg$	Reference
	AIR			
Remote region of Bolivia (air particulate extract)	anthracene, phenanthrene	0.037		179,180,181
	methylanthracene, methyl phenanthrene	0.031		
	fluoranthene	0.041		
	pyrene	0.033		
	benzanthracene, chrysene	0.040		
	benzopyrene, perylene	0.031		
	others			
Antwerp (air particulate extract)	anthracene, phenanthrene	0.89		
	methylanthracene, methylphenanthrene	1.3		
	fluoranthene	0.72		
	pyrene	0.72		
	benzanthracene, chrysene	2.2		
	benzpyrene, perylene	3.0		
	dibenzacridine	0.72		
	others			
Zurich, Switzerland (airborne particulates)	phenanthrene		3,400	182
	anthracene		220	
	fluoranthene		12,900	
	pyrene		18,000	
	benzo[e]pyrene		48,000	
	benzo[a]pyrene		40,500	
	perylene		9,300	
New York City (airborne particulate extract)	isoquinoline	0.18		183,184
	methylisoquinoline	0.31		
	ethylisoquinoline	0.16		
	benzo(f)isoquinoline	0.11		
New York City (airborne particulates)	phenanthrene	0.5		185
	anthracene	0.1		
	fluoranthene	1.9		
	pyrene	2.0		
	perylene	0.2		
	others			
College Park, MD (airborne particulates)	fluoranthene	4.1		186
	pyrene	5.2		
	benz[a]anthracene	4.6		
	chrysene	4.8		
	benzo[e]pyrene	4.6		
	benzo[a]pyrene	3.2		
	benzoperylene	3.9		
Oslo, Norway (airborne particulates)	phenanthrene		160,000	187
	fluoranthene		1,525,000	
	pyrene		1,106,000	
	benzo[a]pyrene		202,000	
	coronene		80,000	
	others			
Simcoe, Canada (airborne particulates)	fluorene			188
	phenanthrene			
	fluoranthene			
	pyrene			
	chrysene			
	benzofluoranthene			
	benzpyrene			
	others			
Urban air particulates, Indiana	120 PAHs identified			189
Air particulates near coke oven plant	anthracene			190
	phenanthrene	130		
	fluoranthene	332		
	pyrene	184		
	chrysene	582		
	perylene	44		
	benzopyrenes	473		
	others			
Coke oven emissions	fluorene		271,000	111
	phenanthrene		86,000	
	anthracene		943,000	
	methylphenanthrene		180,000	
	methylanthracene		1,023,000	
	fluoranthene		6,000,000	
	pyrene		4,600,000	
	chrysene & triphenylene		4,200,000	
	benzo[a]pyrene		2,600,000	
	coronene		865,000	
	many others			
Coal fly ash	anthracene, 9,10-demethylanthracene			109
	benzo(a)pyrene, benzo(e)pyrene			
	benzo(b)phenanthrene, chrysene			
	fluorene, fluoranthene, perylene			
	phenanthrene, pyrene, triphenylene			

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TABLE 9 (continued)

SOIL AND SEDIMENT

Soil--Russia	benzo[a]pyrene	0.1-6	191
Soil--Swiss mountain town:			
center of town	PAH (total)	110,000	192
at highway	PAH (total)	220,000	
open country	PAH (total)	5,000	
Sediment in a stream flowing through an oil tank farm in Knoxville, TN	naphthalene	7,000	176
	alkylnaphthalene	320,000	
	anthracene	3,400	
	benz(a)anthracene	120	
	benz(a)pyrene	48	
River sediment from the Rhondda Farm Valley, South Wales (site of coal mine operated in 19th and early 20th centuries)	anthracene	7,800	91
	fluoranthene	6,500	
	pyrene	5,100	
	2,3-benzofluorene	1,800	
	chrysene	35,500	
	3,4-benzpyrene	7,200	
	1,2,3,4-dibenzanthracene	5,400	
	benzo(ghi)perylene	4,200	
Lake sediments, Greifensee, Switzerland	phenanthrene	340	182
	anthracene	30	
	fluoranthene	420	
	pyrene	380	
	benzo[e]pyrene	210	
	benzo[a]pyrene	160	
	perylene	40	
River sediment near chemical manufacturing plant	fluorene	2,000-10,000	193
	phenanthrene	200-25,000	
	methylphenanthrene	400-20,000	
	fluoranthene	1,000-60,000	
	pyrene	500-75,000	
	others		
Marine Sediment, Buzzards Bay, Mass.	phenanthrene	53	
	fluoranthene	130	102,194
	pyrene	120	102,194
	others		
	aza-arenes	140	
Street dust	phenanthrene	3,500	182
	anthracene	450	
	fluoranthene	10,000	
	pyrene	7,200	
	benzo[e]pyrene	3,900	
	benzo[a]pyrene	2,700	
	perylene	660	
Ash from volcano	benzopyrene	5.4-6.1	164

WATER

Water in a stream flowing through an oil tank farm in Knoxville, TN	naphthalene	8.0	176
	alkylnaphthalenes	850	
	anthracene	3.3	
	benz[a]anthracene	0.19	
	benz[a]pyrene	0.039	
Well in Ames, IA near site at a coal gas plant that operated until 1970's	acenaphthylene	19.3	92
	1-methylnaphthalene	11.0	
	methylindenes	18.8	
	acenaphthene	1.7	
	benzothiophene	0.4	
	others		
Tire manufacturing plan wastewaters	naphthalene	100	142
	1-methylnaphthalene	120	
	phenanthrene	70	
	methylphenanthrene	60	
	fluoranthene	8	
	pyrene	10	
	others		
Thames River	fluoranthene	0.21	139
	pyrene	0.52	
	benzopyrene	0.33	
	perylene	0.43	
	others		
Tap water Switzerland	phenanthrene	0.01	140
	pyrene	0.01	
Tap water, U.S.	benzo[a]pyrene	0.00002-0.002	170
Rain water	phenanthrene		195
	methylphenanthrene		
	fluoranthene		
	pyrene		
	chrysene		
	benzopyrene		
	others		
Tap water, Athens, GA	naphthalene	0.009	151
	dibenzofuran	0.032	
	fluorene	0.031	
	phenanthrene	0.755	
	fluoranthene	0.023	

MIXED-FUNCTION OXIDASE ACTIVITY IN FISH EXPOSED TO POLYNUCLEAR AROMATIC HYDROCARBONS

Polynuclear aromatic hydrocarbons and many other foreign compounds are metabolized by the mixed-function oxidase (MFO) system. Although this group of enzymes is well characterized in mammals[61,196-200], it was not until 1963 that this metabolic capability was suggested in fishes[201]. As in mammals, the fish MFO system has been shown to be inducible. Some of the compounds and mixtures demonstrated to be inducers are: petroleum, PCB's[206-208], 3-methylcholanthrene[209-212], 2,3-benzanthracene[247] and benzo(a)pyrene[213].

Of particular interest from an environmental standpoint has been the finding that fishes exposed to petroleum under laboratory and field conditions show induced MFO activity[198,202-205,207,214]. One MFO enzyme system, aryl hydrocarbon hydroxylase (AHH), has been suggested as an environmental monitor [202] since not only does it respond to the presence of hydrocarbons, but it activates certain PAH's to carcinogenic metabolites.

The current investigation was designed to examine several PAH's to determine whether MFO induction in rainbow trout is a response to particular (especially carcinogenic) PAH's or to general PAH exposure and to evaluate the relationship between the concentration of a recognized carcinogen, benzo-(a)pyrene {B(A)P}, in tissue and hepatic MFO {AHH, aniline hydroxylase (AH) and P-450} induction.

EXPERIMENTAL DETAILS

Bioassay Setup--

Injection experiments--Rainbow trout were obtained from the Genoa National Fish Hatchery, Genoa, Wisconsin, and held in flowing Lake Superior water for six months prior to use. The fish used in the experiments weighed from 30-50 g and had a total length range of 14-17 cm. All fish sampled were sexually immature and were one year old at the start of the six-month experimental period.

Several PAH's were administered in sterile peanut oil by intraperitoneal injection. After anaesthetizing in 100 mg/ℓ MS-222 (ethyl m-aminobenzoate methanesulfonic acid salt), each fish received an 0.2 *mℓ* injection with a 22-gauge needle. The standard dose of B(a)P was approximately 30 mg/kg. B(a)P was given in doses from 3 μg/kg to 300 mg/kg in dose response experiments. Controls received 0.2 *mℓ* injections of peanut oil.

Experiments were carried out in 55-liter stainless steel tanks supplied with 10±1°C Lake Superior water by a Mount-Brungs proportional dilutor, at a rate to give a tank turnover time of 2.5 hr. The photoperiod was 16:8 (light:dark). Fish were fed 3% of their body weight per day Zeigler Bros. 3/16" pellets) during the experiments except on the day they were to be sacrificed. The tanks were siphoned daily.

The water chemistry data during the experiments were: alkalinity 40.8±0.6 mg/ℓ $CaCO_3$, hardness 45.1±0.3 mg/ℓ $CaCO_3$, conductivity 90±3 µmhos/cm, pH 7.05-7.79, and dissolved oxygen 92±5% saturation.

Polycyclic aromatic hydrocarbon flow-through exposures of rainbow trout--

Conditions were similar to those described above. Rainbow trout from the same cohort were exposed to both pyrene (pyr) and fluoranthene (fl) in one experiment and to B(a)P in the other. During each experiment aliquots of a PAH stock solution were diluted 1:1000 with lake water and added daily to a 20-liter reservoir. A control reservoir was filled with 0.1% acetone in lake water. Nominal acetone levels for both control and experimental tanks were 10 µℓ/ℓ (ppm).

During the B(a)P exposure, rainbow trout (1½ yr old) were exposed to solubilized B(a)P in water, with and without the addition of coal particles. Three exposures were run in 55 ℓ stainless steel tanks containing: coal, B(a)P, and coal plus B(a)P. Reservoirs were filled daily with B(a)P stock solutions diluted 1:1000, and ground coal particles (≤0.125 mm). The control (coal alone) reservoir received the same proportion of acetone. Under the two reservoirs containing coal were magnetic stirrers powering 2" Teflon-lined stir bars within the reservoirs. Calculated concentrations were 50 mg ℓ^{-1} for coal and 2 µg ℓ^{-1} for B(a)P.

FMI (Fluid Metering, Inc.) lab pumps with stainless steel fittings and tubing were used to meter reservoir solutions into the tanks. Three water samples were taken during each experiment. Lake Superior water at 10±1°C for the pyr/fl experiment and 13±1°C for the B(a)P experiment was delivered at a rate of 25 ℓ/hr (2.2-hr tank turnover time) and 16.7 ℓ/hr (3.3-hr tank turnover time) respectively.

Fish for enzyme and tissue analyses were taken on days 3,7,10 and 21 for the pyr/fl experiment and on day 10 for the B(a)P experiment.

Preparation of Microsomes--

The trout were killed by cervical dislocation and the livers quickly removed and placed in cold 1.15% KCl. Individual livers were blotted and weighed before homogenizing. Microsomes were then prepared by grinding 2-3 pooled livers in 4 vol 1.15% KCl using a Potter-Elvehjem homogenizer with Teflon pestle. Homogenates were centrifuged at 15,000 G for 20 min and the supernatant spun at 100,000 G for one hour on a Beckman L5-50 ultracentrifuge (0°C). The microsomal pellet was rinsed three times with 1.15% KCl and resuspended in 1.15% KCl by sonicating (Branson Instruments) 3 sec after gentle homogenizing.

During PAH bioaccumulation exposures, 14-20 fathead minnows were assayed for MFO activity. After rinsing with lake water, the fish were minced with scissors, homogenized, and centrifuged as described above. The microsomes from these whole fish homogenates were then used for AHH assays.

Enzyme Assays--

Aryl hydrocarbon hydroxylase (AHH) and aniline hydroxylase (AH) were assayed by literature methods[198,215]. Aliquots of the microsomal preparation containing 0.4-2 mg protein were added to incubation vials (without substrate) and frozen for 24-48 hr until analysis. The incubation mixture for both assays (total vol 0.9 *mℓ*) contained 50 μM TES {N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid} buffer, pH 7.50; 0.6 mg NADPH (sigma, Type I); 3 μM $MgCl_2$, and 200 μℓ microsomal suspension.

The assays were initiated by adding 100 μℓ substrate: 100 nM B(a)P in acetone and 10 μM aniline-HCl. Incubations were carried out in a shaking water bath under dimmed lights. The water bath temperature was 28.5°C[213] and the incubation times were 30 min (AHH) and 20 min (AH). Each sample was measured in duplicate and blanks were run with each set of assays. Hydroxylated B(a)P was measured at 396 nm excitation and 522 nm emission on an Aminco-Bowman spectrophotofluorometer calibrated against 3-hydroxy B(a)P (received through the courtesy of the National Institutes of Health.) For AH determinations the p-aminophenol content was measured at 630 nm on a Beckman DB-G spectrophotometer equipped with a recorder scale expander. Sublimed p-aminophenol was used as the standard.

With the remaining microsomal preparation, cytochrome P-450 concentration was determined after diluting with 25% glycerol in 0.1 M TES buffer, pH 7.40. The CO difference spectrum was measured on a Beckman DB-G spectrophotometer[216]. Protein was determined using a microbiuret method[135]. Crystalline bovine serum albumen was used as the standard. On most samples, only one measurement was made of P-450 concentration on each sample day with replicates generally within 15%.

Tissue Analyses (PAH Exposures: injection and water uptake)--

After the livers were removed for MFO assays, the fish were gutted (kidney remaining), and rinsed with Lake Superior water, methanol, and finally water. Fish from each exposure tank were combined and ground thoroughly in a blender. The homogenized fish were stored in 8-oz glass jars with foil-lined lids at -20°C. Ten grams of tissue from fish exposed to B(a)P (by injection and in water) were extracted and analyzed as described under Section 5. Fish exposed to pyrene and fluoranthene were extracted and analyzed similarly to fish exposed to coal leachate and distillate (see Section 4.)

Water Analysis (PAH Exposures of Rainbow Trout)--

100 mℓ methylene chloride(pyr/fl) or hexane {B(a)P} was placed into a 2 liter volumetric flask followed by 1900 *mℓ* of tank water which had been filtered through a Gelman glass fiber filter (0.2-10 μM). A 1½" Teflon-

coated stirring bar was introduced and the sample extracted by emulsifying the contents for 2 hr. The organic layer was dried over Na_2SO_4 and concentrated for GC analysis in a Kuderna-Danish evaporator. The recovery efficiency for known samples was 95±4%.

Results

Effects of Several Polynuclear Aromatic Hydrocarbons on Mixed-Function Oxidase Activity--

The effects of several aromatic compounds (injected dose 30 mg/kg) on MFO parameters indicated that in general it was the higher molecular weight compounds that caused induction (Figure 8). Cytochrome P-450 levels were elevated by injection with Aroclor 1254 (a PCB mixture), pyrene, chrysene, and B(a)P. The CO difference spectra of induced P-450 did not show the spectral shift to 448 nm characteristic of mammalian systems. This observation is consistent with other studies on piscine P-450 measurements[211,217,233]. AHH activity showed a dramatic 12-fold increase three days after B(a)P administration. Injected Aroclor 1254 also caused significant hydroxylase induction. This latter observation is in agreement with previous studies where PCB's in food have induced AHH activity in coho salmon[207,208] and PCB's in water caused very high AH and N-demethylase activities in channel catfish[206]. The response of AH to injection of PAH's, although lower in magnitude, was generally similar to that for AHH. The exception was chrysene which caused significant AH and P-450 induction but no AHH enhancement.

Rainbow trout were also exposed to three dissolved PAH's under flow-through conditions. Table 10 shows the enzyme and tissue data after exposure to pyrene and fluoranthene (pyr/fl, combined) and to B(a)P. The day 21 (pyr/fl) and day 10 B(a)P enzyme assays are of particular interest. Although the total accumulated pyrene and fluoranthene was over four times that of accumulated B(a)P, the accumulation of pyr/fl did not initiate P-450 or AHH induction. In contrast, accumulated B(a)P caused significant induction of both hydroxylase activities and P-450 content after 10 days.

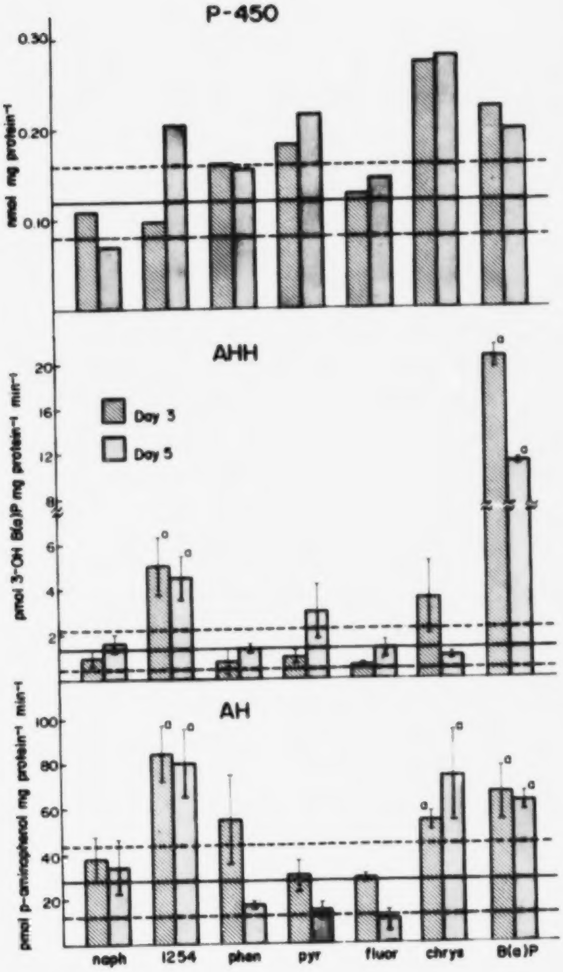
Benzo(a)pyrene Tissue Levels and Mixed-Function Oxidase Induction--

The specific effects of the known MFO inducer B(a)P were examined by comparing B(a)P tissue levels with concomitant MFO measurements in a dose-response injection experiment and in a water uptake experiment. In the dose-response study, significant induction of both AHH and AH activities was found at an injected B(a)P dose of 300 μg/kg and above (Figure 9). The cytochrome P-450 concentration at the 300 μg/kg dose on day 5 was elevated. Therefore, the 300 μg/kg dose appears to be an approximation of a minimum effective dose (MED) for MFO induction in rainbow trout. The AH results should be contrasted with those of Payne[130] who found no basal or inducible AH activity in rainbow trout.

Gutted fish from the dose-response experiment were analyzed for B(a)P 3 and 5 days after injection (Table 11). The measured B(a)P concentrations were generally quite different from the injected dose perhaps because of different rates of absorption from the peritoneal cavity, weight variations

FIGURE 8

EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)
ON MICROSOMAL ENZYMES IN RAINBOW TROUT[a]



[a] The two bars for each compound are values obtained 3 and 5 days after i.p. injection of 30 mg/kg PAH. For each compound tested, the three enzyme assays were done using the microsomal fraction obtained from 2-3 pooled livers. AHH and AH assays were done in duplicate with the error bars representing the standard deviation. The mean and standard deviation of seven control fish samples are indicated by the horizontal lines. a=$p < 0.05$

abbreviations: naph, 1,2,4-trimethylnaphthalene; 1254, Aroclor 1254; phen, phenanthrene; pyr, pyrene; fluor, fluoranthene; chrys, chrysene; B(a)P, benzo(a)pyrene.

TABLE 10

PAH TISSUE CONCENTRATIONS AND MFO MEASUREMENTS DURING WATER UPTAKE EXPERIMENTS[a]

PAH Concentration (μg/kg)		Time (day)	P-450[b] (nM/mg protein)	AHH (pM/mg protein/min)	AH (pM/mg protein/min)
pyr	fl				
95±8	318±10	3	0.083	2.18 ±0.43	---
82±7	273±14	7	0.107	1.18 ±0.27	---
150±11	408±20	10	0.103	1.74 ±0.02	---
281±23	1,250±38	21	0.071	2.50 ±0.43	---
0 (controls, n=6)	0		0.107 ±0.048	1.72 ±0.80	---
B(a)P					
368±24		10	0.238* ±0.018	3.80* ±0.62	34.52* ±5.14
73.1±24[c]		10	0.115 ±0.028	1.66 ±0.88	11.47 ±1.13
0 (controls, n=2)			0.175 ±0.032	1.76 ±0.25	20.01 ±3.21

[a] The mean water concentrations during the exposures were 3.89 ±0.08 μg l^{-1}, pyrene; 3.31 ±0.08 μg l^{-1}, fluoranthene; and 0.40 ±0.21 μg l^{-1}, B(a)P. Control values were obtained by taking several (n) 3-fish samples from control tanks at various times during the experiments. Induced enzyme levels ($P < 0.05$) are indicated by asterisks.

[b] During the pyr/fl experiment only one measurement of P-450 was made on microsomes prepared from 3 pooled livers; duplicates were run during the B(a)P exposure.

[c] The exposure tank contained 50 mg l^{-1} ground coal (≤0.125 mm) in addition to B(a)P. Water concentrations of B(a)P determined on filtered samples were <0.1 μg l^{-1}.

among the fish, different rates of metabolism, and injection variations.

In the water uptake experiment (Table 10), exposure of trout to 0.4 μg/l B(a)P resulted in accumulation of 368 μg/kg B(a)P after ten days. Table 10 also shows the corresponding enzyme data, which demonstrated induction of all three MFO parameters at that time. Trout containing 73 μg/kg B(a)P showed no MFO induction after a 10-day exposure to B(a)P plus coal particles {low levels of B(a)P (<0.1 μg/ℓ)}.

To determine whether there was a correspondence between accumulated B(a)P and MFO induction, the B(a)P analyses from the injection experiment (Table 2) and from the water uptake experiment (Table 10) were compared to their corresponding MFO measurements (Figure 9, Table 10). From the combined results (Table 12), it appears that although there was one exception (i.e., a lack of AHH induction at 350 μg/kg), B(a)P levels above 300 μg/kg were accompanied by induced MFO activity.

Discussion

Mixed-function Oxidase Induction by Various Polycyclic Aromatic Hydrocarbons--

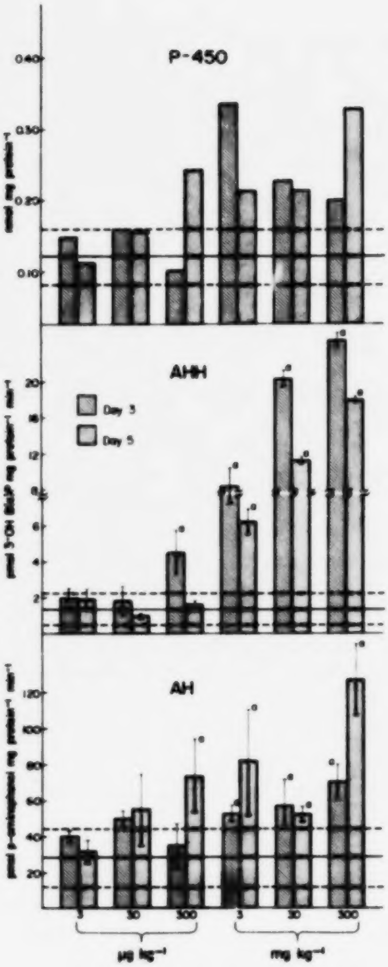
The MFO system in rainbow trout was found to be inducible only by certain compounds.

Of the compounds tested, chrysene, B(a)P, and Aroclor 1254 are classified as carcinogenic by NIOSH[218]. The injection experiments showed that P-450 and the hydroxylases responded to these compounds. The PAH water uptake experiments also indicated a degree of specificity of the MFO system since accumulated B(a)P caused induction, but tissue levels of pyrene and fluoranthene (combined) did not elicit such a response. This lends some support to the rationale behind using MFO levels, such as AHH, as indicators of environmental exposure to carcinogens. The situation is confounded, however, because of possible synergistic interactions among the wide array of watersoluble organic compounds. For example, the long chain hydrocarbon n-dodecane greatly enhanced the carcinogenic potency of B(a)P in mice skin tumor studies[219]. Also some compounds, such as the synthetic β-naphthoflavone, cause MFO induction but are not carcinogenic[220].

Short term injection experiments have shown to be a rapid means of screening PAH's for MFO inducibility. However, even with pooled samples, large variations can occur, and the need for adequate sampling is recognized. Additionally, the physiologically abrupt absorption of compounds from the peritoneal cavity may augment the MFO response. For example, injection of pyrene caused P-450 elevation, yet throughout the 21-day water exposure to pyrene (with fluoranthene), there was no P-450 induction. The relatively low bioaccumulation potential of pyrene is demonstrated by the accumulation of only 281 μg/kg after 21 days. This may explain the lack of MFO response and points out the importance of determining bioconcentration factors[221] and depuration rates when assessing possible carcinogenic impact. Therefore, it appears that through the use of i.p. injections for initial screening coupled with bioaccumulation exposures, discrete compounds and components of complex mixtures could be tested for relative MFO inducibility in fishes.

FIGURE 9

DOSE RESPONSE FOR HEPATIC MICROSOMAL ENZYMES IN RAINBOW TROUT[a]



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[a] Each fish was injected i.p. with (B(a)P and the MFO response measured 3 and 5 days later. For each dose the three enzyme assays were done using the microsomal fraction obtained from 2-3 pooled livers. AHH and AH activities were measured in duplicate with the error bars representing the standard deviation. The mean and standard deviation of seven control fish samples are indicated by the horizontal lines. a = $p < 0.05$

TABLE 11

TISSUE ANALYSES FOR DOSE-RESPONSE EXPERIMENT[a]

Approximate Injected Dose (µg/kg)	B(a)P Concentration In Tissue (µg/kg)	
	Day 3	Day 5
30,000	3260±50	458±31
3,000	320±54	537±65
300	249±33	350±21
30	130±27	140±14
3	<30	<30

[a] The B(a)P doses indicated were injected i.p. in peanut oil and tissue concentrations measured 3 and 5 days later in 2-3 gutted fish (pooled). The minimum detectable B(a)P level was 30 µg/kg.

TABLE 12

RELATIONSHIP BETWEEN B(a)P CONCENTRATION IN RAINBOW TROUT AND MFO INDUCTION[a]

	P-450	AHH	AH
3260±50 (i)	+	+	+
537±65 (i)	+	+	+
458±31 (i)	+	+	+
368±24 (w)	+	+	+
350±21 (i)	+	-	+
320±54 (i)	+	+	+
249±33 (i)	-	+	-
140±14 (i)	-	-	-
130±27 (i)	-	-	-
73±24 (w)	-	-	-
<30 (i)	-	-	-

[a] These data were compiled from both the injection experiment [i, Table 2, Figure 9] and the water uptake experiment (w, Table 1). Pooled livers were analyzed for MFO activity and the corresponding gutted fish analyzed for B(a)P. A plus (+) indicates enzymes in induced state.

Effects of Coal on Benzo(a)pyrene Uptake--

The presence of coal particles reduced the total accumulation of B(a)P to 20% of that accumulated by rainbow trout exposed to B(a)P without particulates. Ground coal at a concentration of 50 mg/ℓ (particle size ± 0.125mm) lowered the exposure concentration of B(a)P from 0.4 μg/ℓ to below detection (0.1 μg/ℓ)in filtered water samples. The observed accumulation in the presence of coal particles indicated that B(a)P adsorbed onto particulates might be accumulated at gill and/or gut membranes. Uptake from particulates may be an important route of accumulation in the aquatic environment because the major portion of PAH's are associated with suspended solids[144].

Relationship of Mixed Function Oxidase Levels to Concentrations of a Specific Carcinogen {Benzo(a)pyrene} in Tissue of Rainbow Trout--

The few reported environmental concentrations of B(a)P in fishes have been low. For example, Pancirov and Brown[222] found only 1.5 μg/kg in menhaden and <1 μg/kg in flounder and cod fish caught off the New Jersey coast.

We have, however, demonstrated that B(a)P can be readily bioaccumulated from water by rainbow trout. Although the concentration of B(a)P used in the water uptake experiment (0.40 ± 0.21 μg/l) is high compared to ground water concentrations (0.0001 μg/l)[161], it was less than the concentrations from several metropolitan raw water supplies in the U.S. (1-2 μg/l)[223], and downriver from a petroleum industry in Russia (0.05-3.5 μg/l)[224]. The observed bioconcentration in rainbow trout should be contrasted with a study by Lu[225] who found that B(a)P was rapidly metabolized by mosquitofish (*Gambusia*) in water uptake experiments and was appreciably bioaccumulated only by food chain transfer and in the presence of an MFO inhibitor.

The hitherto unknown relationship between hepatic MFO activity and B(a)P concentration in tissue has been explored in this investigation. In Table 12, the B(a)P tissue concentrations from Table 11 were compared to their respective enzyme levels in Figure 9. Also included were the B(a)P water exposure data from Table 10. From this study it appears that induced MFO activity might be predicted in fish having B(a)P tissue burdens exceeding 300 μg/kg.

BIOACCUMULATION OF POLYNUCLEAR AROMATIC HYDROCARBONS

The accumulation of aromatic hydrocarbons from water[171,226,227] and food[226,228,229] by a variety of aquatic organisms[161,226,230] including algae, plankton, mollusks, worms, clams, and fish (Tables 13 and 14) has generated some interest[231]. Organisms containing accumulated aromatic compounds may be involved in food-chain biomagnification processes which may result in increased concentration of hydrocarbons per weight of tissue at successively higher trophic levels, perhaps including humans[101,171,226,231,232]. Also aromatic hydrocarbons, accumulated in higher organisms containing mixed-function oxidase enzymes, might be transformed by these enzymes into metabolites capable of producing adverse effects on the organism or its progeny [130,217,229,231,233,234]. These concerns are apparently justified by the observation of increased tumor frequency in fish living in polluted water containing PAH (e.g., 10-50 μg/ℓ benzanthracene[235]) with respect to fish living in relatively unpolluted water[235-237]. Although it is not known if PAH were involved in the tumor production, it is known that certain PAH are capable of producing tumors in laboratory fish[238].

Aquatic organisms, such as *Tubifex* worms and snails, which do not contain the enzyme system necessary for metabolism of aromatic compounds, tend to concentrate these compounds to higher levels (per weight of tissue) than do organisms such as fish, which contain the necessary enzymes[130,226,225]. In organisms of the latter type, it has been noted that in some cases the presence of certain amounts of PAH and other compounds may lead to increased levels of PAH metabolic activity[130,204,205,207,231] (i.e. "MFO induction"). The relationship between increased enzyme activity and levels of accumulation of polycyclic aromatic compounds in a particular organism has not been studied extensively, but it appears that the presence of inducers of mixed-function oxidases may raise metabolism and lower accumulation of PAH in the organism and that the presence of an inhibitor may lower the metabolism and raise the accumulation[212,225].

The goal of the present study was to provide data, which are presently scarce, on the potential for accumulation of various polycyclic aromatic compounds in fish and to determine if any relationship exists between the mixed-function oxidase activity in the fish and the levels of accumulation.

The experimental determination of the potential for a given compound to accumulate in an aquatic organism usually involves the derivation of a bioconcentration factor*[221] by direct measurement of the concentration of a compound in the fish and in the water at equilibrium under flow-through conditions.

$$^{*}\text{Bioconcentration factor} \equiv \frac{\text{concentration of compound in tissue}}{\text{concentration of compound in water}}$$

TABLE 13

LEVELS OF VARIOUS AROMATIC COMPOUNDS IN AQUATIC ORGANISMS IN THE ENVIRONMENT

RESULTS FROM SELECTED LITERATURE REPORTS

Compound	Organism	Location	Concentration in organism (mg compd/ kg wet weight)	Tissue analyzed	Concentration in water (μg/kg)	Bioconcentration Factor[a] (time)	Ref.
benzo(a)pyrene	mussel	on creosoted pilings	0.049±0.015	whole organism[b]			244
benzo(a)anthracene	oyster	Long Island Sound	0.008	whole organism[b]			
benzo(a)pyrene	oyster	L.I. Sound	0.002	whole organism[b]			222
pyrene	oyster	L. I. Sound	0.058	whole organism[b]			
methylpyrene	oyster	L. I. Sound	0.011	whole organism[b]			
fluorene	clam		0.046	whole organism[b]			
phenanthrene	clam		0.88	whole organism[b]			245
methylphenanthrene	clam		0.56	whole organism[b]			
benzo(a)pyrene	mussel	S.California	0.0023				237
benzo(a)pyrene	shellfish	?Maine	0.016	whole organism[b]			246
benzo(ghi)perylene			0.025	whole organism[b]			
benzo(a)pyrene	mussel	S.California on pilings	0.008	whole organism[b]			247
benzo(a)pyrene	mussel	new creosoted timbers	0.045	whole organism[b]			248
benzo(a)pyrene	mussel	Vancouver	0.215	whole organism[b]			249
benzo(a)anthrene	crab	Raritan Bay, New Jersey	0.002	whole organism			222
benzo(a)pyrene			0.003				
pyrene			0.006				
methylpyrene			0.002				
benzo(a)pyrene	menhaden	Raritan Bay, New Jersey	0.002	whole organism			222
pyrene	flounder	Long Island	0.002	whole organism			222
methylpyrene	flounder	Long Island	0.0005	whole organism			
total PAH	oysters	Gulf of Mexico	2 - 9	whole organism			250
Aroclor 1254	burbot	W. Lake Superior	1.4±0.4	whole organism[c]	.0008	1.75×10^6	
	lake trout	"	1.8±1.6	whole organism[c]	.0008	2.25×10^6	251
	long-nose sucker	"	0.9±0.8	whole organism[c]	.0008	1.13×10^6	
	slimy sculpin	"	0.34	whole organism[c]	.0008	4.25×10^5	
	mysid	"	0.085±0.029	whole organism[c]	.0008	1.06×10^5	
polychlorinated biphenyls	fish	Puget Sound	0.84	whole organism[c]			252
	mussels		0.21				

[a] Bioconcentration factor = (concentration of compound in fish in μg/kg)/(concentration of compound in water in μg/kg).

[b] shucked

[c] composites

TABLE 14

TABULATION OF LEVELS OF VARIOUS AROMATIC COMPOUNDS IN AQUATIC ORGANISMS IN LABORATORY EXPERIMENTS RESULTS FROM SELECTED LITERATURE REPORTS

Compound	Organism	Conc. In Organism (mg/kg)	Tissue Analyzed	H_2O Conc. (µg/ℓ)	Bioconc. Factor (time)^{a}	Reference
anthracene^{c}	*Daphnia pulex*	0.015*	whole organism	0.02	760 (24 hrs)	241
naphthalene^{c}	polychaete	∼5.5	whole-body males	20	275*(20 hrs)	253
naphthalene^{c}	polychaete	∼4.2	whole-body females	27	156*(20 hrs)	
naphthalene^{d}	clam	0.43±0.01	whole organism(shucked)	71	6.1 (24 hrs)	
phenanthrene^{d}	clam	2.8±1.1	whole organism(shucked)	89	32.0 (24 hrs)	
chrysene^{d}	clam	0.54±0.3	whole organism(shucked)	66	8.2 (24 hrs)	45
benzo(a)pyrene^{d}	clam	0.45±0.1	whole organism(shucked)	52	8.7 (24 hrs)	
naphthalene^{d}	sheepshead minnow	60	whole organism ?	1000	60*(4 hrs)	254
1-methylnaphthalene^{d}	sheepshead minnow	205		1000	205*(4 hrs)	
naphthalene^{d}	clam	1.9	whole organism(shucked)	840	2.3 (24 hrs)	
1-methylnaphthalene^{d}	clam	2.9	whole organism(shucked)	340	8.5 (24 hrs)	
2-methylnaphthalene^{d}	clam	3.9	whole organism(shucked)	480	8.1 (24 hrs)	46
di-methylnaphthalene^{d}	clam	4.1	whole organism(shucked)	240	17.1 (24 hrs)	
tri-methylnaphthalene^{d}	clam	0.8	whole organism(shucked)	30	26.7 (24 hrs)	
benzo(a)pyrene^{c}	clam	7.2	whole organism(shucked)	30.5	236 (24 hrs)	
benzo(a)pyrene^{c}	mosquito larva	0.0942	whole organism	2.5	2177 (3 days)	
benzo(a)pyrene^{c}	snail	5.1523	whole organism	2.5	37 (3 days)	225
benzo(a)pyrene^{c}	mosquito fish	0.0	whole organism	2.5	0 (3 days)	
biphenyl^{e}	rainbow trout		muscle		438±38 (∞)	240
Aroclor 1254^{f}	cockle	40.8	whole organism(shucked)	250	163 (40 days)	255
Aroclor 1254^{f}	tellin	34.5	whole organism(shucked)	250	138 (40 days)	
heptachlor^{e}	spot	0.308	whole organism	0.14	2,200 (72 hrs)	256
trans-Chlordane^{e}	spot	0.132	whole organism	0.04	3,300 (72 hrs)	
Aroclor 1254^{e}	spot	27	whole organism	1	27,000 (56 days)	257
Aroclor 1254^{e}	spot		whole organism			
heptachloronorbornene^{e}	fathead minnow	448	whole organism	40	11,200 (30 days)	258
pentachlorophenol^{c}	rainbow trout	16	liver	26	615 (24 hrs)	
		6.5	blood	26	250 (24 hrs)	259
		6.0	fat	26	231 (24 hrs)	
		1.0	muscle	26	39 (24 hrs)	
pentachloroanisole^{c}	rainbow trout	3.2	liver	24	133 (12 hrs)	
		1.0	blood	24	47 (12 hrs)	259
		85.0	fat	24	3,542 (12 hrs)	
		2.3	muscle	24	96 (12 hrs)	
p-dichlorobenzene^{e}	rainbow trout		muscle	1.6±0.2	215±21 (∞)	240
2,2^{1},4,4^{1}-tetra-chlorobiphenyl^{e}	rainbow trout		muscle		9850±2890 (∞)	239

[a] Bioconcentration factor ≡ (concentration of compound in fish in µg/kg)/(concentration of compound in water in µg/kg)

[b] Value calculated for data in article [c] static, ^{14}C [d] static [e] flow through [f] renewed static

Other methods for the bioconcentration factor derivation include indirect approaches based on pharmacokinetic models[221,232,239] or estimates based on partition coefficients[221,232,240]. In studies involving measurements[225,229,241], ^{3}H- or ^{14}C-labeled compounds are most often employed and metabolites are usually, but not always[242], separated from the parent compound prior to radioactivity determination.

For the present study the direct measurement approach was employed in which the concentration of a polycyclic aromatic hydrocarbon in the exposure water and the fish tissue was measured by HPLC-GC procedures (Section 5) over a four week uptake period and a one week depuration period. The levels of activity of the mixed function oxidases in the fish were also monitored.

Experimental Details

Aquaria--

Forty-liter glass tanks were equipped with a Masterflex pump (Model 7015, Cole-Parmer) to deliver 210 mℓ/min of Lake Superior water and an FMI (Fluid Metering, Inc.) laboratory pump to deliver 2.1 mℓ/min of an aqueous methanol solution of polynuclear aromatic hydrocarbons. The tank volume of 36 ℓ water, which contained ∿10 μℓ methanol per liter of water, was turned over every 3.2 hr. The temperature was maintained at $24 \pm 1^{\circ}$C and the photoperiod was 16:8. Tanks were cleaned daily.

Fish--

The fish used for these studies were five- to six-week-old fathead minnows which were reared in Lake Superior water at the United State Environmental Protection Agency Environmental Research Laboratory, Duluth, Minnesota. They were fed a maintenance diet of ∿2% of their body weight of #1 pellets (Zeigler Bros.) and live brine shrimp nauplii daily.

Procedure--

The fish (250-300) were exposed to lake water containing PAH for 28 days and then to only lake water for an additional five days. Sixteen to twenty fish samples were removed on various days during the experiment, rinsed with Lake Superior water, and blotted dry on an absorbent towel. Analysis of the PAH content of the fish then proceeded as described in the analytical section (Section 5) using the Styragel-HPLC/GC-PID procedure. Determination of the mixed-function oxidase (MFO) activity of whole fish homogenates was carried out on days 7, 14, and 28, using methods described in the mixed-function oxidase discussion (Section 6). One to four liters of tank water was also analyzed on various days as described in Section 5 using the "pre-column" concentration/pre-column coupled-reverse phase HPLC/GC-PID procedure.

The analysis of the β-naphthoflavone in water and fish tissue in the presence of phenanthrene (experiment #2) required some modifications of the original analytical techniques because this compound could not be determined by GC. For the analysis in water, the usual pre-column concentration/pre-column coupled reverse phase HPLC procedure was employed. The ultraviolet absorbance of the resulting HPLC fraction, which contained phenanthrene and β-naphthoflavone, was determined at 270 and 255 nm. Using Beer's Law plots for each of the compounds at each of the wavelengths, the concentration of the phenanthrene and β-naphthoflavone in the water could be calculated. For the analysis of the β-naphthoflavone in fish tissue, the usual styragle-HPLC fractionates produced a methylene chloride fraction containing the flavone (but not phenanthrene) which was analyzed by reverse phase HPLC. The conditions for the latter analysis were 10% to 90% acetonitrile in water in 30 min at 1.5 mℓ/min total flow with a 5 μ Lichrosorb C-18 column.

Calculation of Bioconcentration Factors--

The calculation of the bioconcentration factors and associated errors (presented in Table 15) were carried out as follows:

$$\mathrm{BCF} = \frac{\mathrm{FCN(I)}}{\mathrm{MWCN(L)}} \pm \mathrm{EBCF(I)}$$

where BCF(I) = bioconcentration factor for day I
FCN(I) = mg of compound per kg fish on day I (Table 16)
MWCN(I) = mean concentration of compound in water up to and including day I (Table 17)
EBCF(I) = maximum error involved in determination of BCF(I) on day I

$$\mathrm{MWCN(I)} = \frac{\sum_{j=1}^{n} \mathrm{WCN}_j}{n}$$

$$\mathrm{EBCF(I)} = \left| \left(\frac{\mathrm{FCN(I)} + \mathrm{EFCN(I)}}{\mathrm{MWCN(I)} - \mathrm{EMWCN(I)}} \right) - \mathrm{BCF(I)} \right|$$

where j = water sample number (1, 2, 3....n)
n = number of water samples taken up to and including day I
WCN_j = concentration of compound in tank water for j^{th} sample
EFCN(I) = error involved in determination of FCN(I) (see Appendix B)
and

$$\mathrm{EMWCN(I)} = \text{error in } \mathrm{MWCN(I)} = \sqrt{\frac{\sum_{j=1}^{n} \{\mathrm{WCN}_j(\mathrm{I})\}^2 - \sum_{j=1}^{n} \mathrm{WCN}_j(\mathrm{I})^2}{n - 1}}$$

Determination of Particulate PAH Load--

In order to determine if any PAH material was associated with particulate matter (11.4 mg/ℓ) in the fish exposure tank of experiment #3, Tank #3, the GF/F filter, used in the water analysis apparatus, (see Section 5) was

TABLE 15

SUMMARY OF BIOCONCENTRATION FACTORS (BASED ON TOTAL WET WEIGHT OF FISH)

Exp #	Tank #	Compound	Bioconcentration factor = (μg PAH/kg fish)/(μg PAH/kg water)[a] Day #1	Day #2	Day #4	Day #7	Day #10	Day #14	Day #18	Day #21	Day #25	Day 28 Trial #1	Day 28 Trial #2	MFO Activity	% Lipid ± S.D.
1	1	dibenzofuran	260± 50	540± 70	1,200± 200	1,200± 200		1,100± 150		1,000± 200		1,100± 160		No	4.8±1.5
		fluorene	250± 50	500± 60	1,200± 100	1,200± 100		870± 100		1,100± 200		1,100± 200			
	2	9-chlorophenanthrene	1,140± 80	1,200± 240	1,900± 1,200	1,700± 1,000		3,200± 1,500		1,200± 600		5,000± 2,000		Yes	4.1±0.6
2	1	phenanthrene	990± 100	800± 700	800± 700	1,300± 900	1,500± 750				1,900± 1,100	2,500± 1,300		Yes	4.8±1.5
		β-naphthoflavone					<90± 40				<100± 80	<100± 40			
	2	phenanthrene	2,000± 300	1,700± 200	2,000± 250	2,300± 200	3,300± 400		3,700± 700		4,200± 1,000	5,100± 1,600		No	3.8±0.7
3	1	phenanthrene	2,000± 200	2,400± 600	1,600± 900	1,800± 800		1,900± 600		2,100± 600		2,000± 600	2,800± 750	Yes	4.1±0.5
		9-chlorophenanthrene	4,400± 600	6,100± 1,000	6,400± 11,000	5,600± 5,000		5,600± 2,000		5,100± 2,000		5,100± 2,000	6,700± 3,000		
	2	phenanthrene			1,500± 200	1,400± 400		1,300± 400		2,000± 600		3,100± 1,000	3,000± 1,000	No	4.3±1.0
	3	dibenzofuran		1,400± 350	830± 200	860± 300		1,800± 900		1,200± 500		1,700± 600	1,600± 500	Yes	4.4±0.9
		fluorene		1,200± 400	830± 300	1,100± 500		2,200± 1,000		1,300± 500		1,800± 800	1,500± 400		
		phenanthrene		2,000± 300	2,000± 200	2,000± 600		3,100± 600		2,200± 600		1,900± 500	2,200± 400		
		1-methylphenanthrene		1,200± 400	1,400± 500	1,300± 400		2,100± 700		1,800± 600		700± 300	1,240± 400		
		fluoranthene		1,600± 300	2,600± 700	4,000± 600		3,200± 800		3,600± 1,000		1,500± 500	1,900± 900		
		pyrene		1,200± 250	1,200± 400	1,400± 700		2,300± 1,000		2,600± 1,000		970± 500	600± 600		

[a] calculated from data presented in Tables 16 and 17.

[b] $\frac{\mu g\ PAH}{kg\ water}$ = mean concentration of PAH determined from all analyses performed up to and including the day of fish sampling.

TABLE 16

SUMMARY OF FISH ANALYSES (BASED ON TOTAL WET WEIGHT OF FISH)

Exp #	Tank #	Compound	Concentration of PAH in Fish: mg PAH/kg fish													Depuration			Mean µg PAH/ℓ water
			Day #1	Day #2	Day #4	Day #7	Day #10	Day #14	Day #18	Day #21	Day #25	Day #28	Day #28 Trial #2	Day #29	Day #30	Day #31	Day #32	Day #35	
1	1	dibenzofuran	0.96± 0.19	1.97± 0.26	4.00± 0.19	4.03± 0.34		3.49± 0.17		3.37± 0.22		3.47± 0.18				<0.1		<0.1	3.19±0.26
		fluorene	0.93± 0.19	1.87± 0.22	4.15± 0.19	4.45± 0.26		3.16± 0.11		3.93± 0.32		3.90± 0.22				<0.1		<0.1	3.45±0.38
	2	9-chlorophenanthrene	0.87± 0.05	0.85± 0.07	1.13± 0.08	1.02± 0.06		1.81± 0.13		0.66± 0.10		2.70± 0.23						<0.1	0.55±0.12
2	1	phenanthrene	4.03± 0.22	2.50± 0.21	2.52± 0.20	3.76± 0.42	4.44± 0.31				4.90± 0.43	6.43± 0.22		2.75± 0.19	0.59± 0.08		<0.1		2.63±0.83
		β-naphthoflavone	<0.05	<0.05		<0.05	<0.16				<0.18	<0.19		<0.42					1.82±0.18
	2	phenanthrene	5.14± 0.53	4.31± 0.24	4.96± 0.81	5.98± 0.20	8.65± 0.58		10.06± 0.42		10.99± 0.73	12.90± 1.04		5.97± 0.43	1.84± 0.18		0.49± 0.10		2.55±0.44
3	1	phenanthrene	3.79± 0.07	4.52± 0.65	3.88± 0.32	4.72± 0.40		5.12± 0.28		5.63± 0.49		5.10 ± 0.33	7.13± 0.31	1.92± 0.31	<0.1		<0.1		2.53±0.44
		9-chlorophenanthrene	3.79± 0.21	5.27± 0.48	9.16± 1.64	8.27± 1.01		8.69± 0.44		7.50± 0.68		7.23 ± 0.43	9.46± 0.71	4.30± 0.44	1.73± 0.48		<0.1		1.41±0.34
	2	phenanthrene			3.38± 0.24	3.35± 0.39		3.14± 0.37		5.05± 0.50		7.19± 0.29	7.05± 0.52	1.61± 0.27	<0.1				2.34±0.54
	3	dibenzofuran		3.17± 0.59	1.93± 0.39	2.31± 0.18		4.27± 0.32		2.73± 0.22		4.02± 0.30	3.81± 0.23	1.26± 0.07	<0.1				2.34±0.51
		fluorene		3.06± 0.67	2.05± 0.40	3.10± 0.32		5.33± 0.33		3.21± 0.22		4.38± 0.70	3.62± 0.17	1.32± 0.07	<0.1				2.49±0.48
		phenanthrene		4.54± 0.29	4.62± 0.06	4.13± 0.26		6.75± 0.27		4.88± 0.67		4.20 ± 0.47	4.92± 0.29	1.55± 0.28	<0.1				2.20±0.25
		1-methylphenanthrene		1.50± 0.41	1.83± 0.45	1.46± 0.14		2.78± 0.24		2.35± 0.28		0.96 ± 0.19	1.70± 0.24	0.14± 0.21	<0.1				1.38±0.19
		fluoranthene		1.93± 0.25	3.08± 0.65	4.42± 0.18		4.01± 0.16		4.66± 0.42		2.04 ± 0.29	2.55± 0.62	0.76± 0.16	<0.1				1.35±0.20
		pyrene		1.10± 0.09	1.04± 0.18	1.36± 0.20		2.56± 0.35		2.80± 0.35		1.14± 0.24	0.69± 0.41	<0.1	<0.1				1.18±0.27

TABLE 17

BIOCONCENTRATION EXPERIMENTS: SUMMARY OF WATER ANALYSIS

	Exp. 1			Exp. 2			Exp. 3								
	Tank 1		Tank 2	Tank 1		Tank 2	Tank 1		Tank 2	Tank 3					
Compound	dibenzo furan	fluorene	9-chlorophenanthrene	phenanthrene	β-naphthoflavone	phenanthrene	phenanthrene	9-chlorophenan threne	phenanthrene	dibenzofuran	fluorene	phenanthrene	1-methylphenan threne	fluorene	pyrene
Day															
0	3.65± 0.02	3.72± 0.01	0.76± 0.01	4.08± 0.22	1.82± 0.26	2.55± 0.14									
1							1.92± 0.14	0.86± 0.06							
2			0.66± 0.01	2.22± 0.24	1.82± 0.19				2.13± 0.14	2.32± 0.13	2.46± 0.22	2.28± 0.14	1.30± 0.10	1.19± 0.04	0.89± 0.09
3	3.15± 0.03	3.49± 0.01													
4			0.37± 0.05			2.74± 0.21	2.98± 0.17	1.99± 0.32	2.28± 0.10						
5										2.40± 0.16	2.56± 0.18	1.66± 0.18	0.91± 0.05	0.98± 0.07	0.78± 0.21
6							3.07± 0.08	1.63± 0.10	2.67± 0.23						
7	3.13± 0.14	3.78± 0.07		2.48± 0.17	1.83± 0.20					3.35± 0.34	3.68± 0.43	2.39± 0.14	1.23± 0.07	1.16± 0.06	1.19± 0.07
8			0.47± 0.02				2.76± 0.09	1.60± 0.07	2.07± 0.25						
9	3.49± 0.15	3.95± 0.07		2.86± 0.13	1.76± 0.22					2.70± 0.16	2.06± 0.08	2.17± 0.09	1.44± 0.04	1.20± 0.15	1.06± 0.13
10			0.60± 0.08			2.47± 0.14									
11							3.26± 0.15	1.82± 0.12	2.99± 0.08						
12										1.39± 0.08	1.73± 0.08	2.49± 0.09	1.36± 0.06	1.38± 0.05	1.32± 0.09
13							2.17± 0.18	1.46± 0.28	2.26± 0.16						
14	2.99± 0.46	3.28± 0.22								1.96± 0.10	2.28± 0.12	2.30± 0.11	1.55± 0.11	1.57± 0.15	1.54± 0.15
15			0.45± 0.08	3.51± 0.31	1.71± 0.22		2.38± 0.17	1.23± 0.08	2.21± 0.17						
16	3.05± 0.28	3.42± 0.32				3.27± 0.70				2.28± 0.12	2.34± 0.13	2.19± 0.11	1.50± 0.11	1.49± 0.10	1.03± 0.07
17			0.60± 0.08												
18				2.29± 0.26	1.66± 0.18		2.79± 0.16	1.22± 0.16	2.93± 0.17						
19										2.35± 0.08	2.52± 0.08	2.09± 0.17	1.44± 0.37	1.35± 0.25	0.95± 0.10
20							2.75± 0.17	1.46± 0.08							
21						2.80± 0.50			3.13± 0.15						
22			0.50± 0.02	2.07± 0.10	1.74± 0.15		2.40± 0.13	1.38± 0.11		2.43± 0.10	2.50± 0.13	2.34± 0.13	1.33± 0.10	1.47± 0.12	1.44± 0.10
23						2.04± 0.08			2.23± 0.11						
24	3.19± 0.21	2.78± 0.15		1.50± 0.07	2.24± 0.17		2.06± 0.22	0.97± 0.12		2.09± 0.20	2.56± 0.14	2.42± 0.15	1.59± 0.11	1.53± 0.16	1.58± 0.10
25			0.47± 0.06												
26									1.99± 0.15						
27							2.42± 0.14	1.07± 0.14		2.51± 0.22	2.57± 0.14	1.86± 0.14	1.49± 0.11	1.57± 0.13	1.21± 0.09
28	2.87± 0.44	3.16± 0.26				2.01± 0.08	1.91± 0.18	1.68± 0.19	1.14± 0.20						
Mean ± Std Dev.	3.19± 0.26	3.45± 0.38	0.54± 0.12	2.63± 0.83	1.82± 0.18	2.55± 0.44	2.53± 0.44	1.41± 0.34	2.34± 0.54	2.34± 0.51	2.48± 0.48	2.20± 0.25	1.38± 0.19	1.35± 2.20	1.18± 0.27

TABLE 18

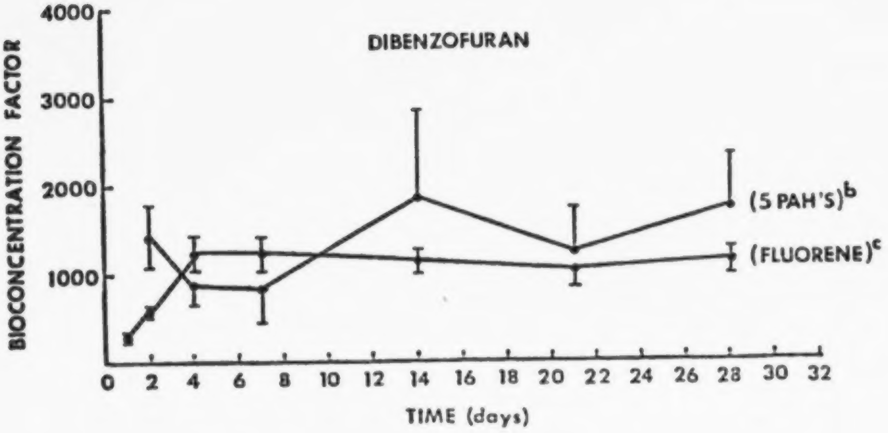
AHH ACTIVITY IN FATHEAD MINNOWS EXPOSED TO PAHs[a]

Exp #	Tank #	Compound	AHH Activity (pM 3-OH-BaP)/(mg protein/min)		
			Day 7	Day 14	Day 28
1	1	dibenzofuran fluorene		0.23±0.02	0.30±0.04
	2	9-chlorophenanthrene		0.21±0.09	1.0±0.10
2[b]	1	phenanthrene β-naphthoflavone	1.0±0.4	1.0±0.5	0.69±0.04
	2	phenanthrene	0.31±0.02	0.4±0.1	0.44±0.02
3	1	phenanthrene 9-chlorophenanthrene	0.37±0.08	1.5±0.2	1.3±0.3
	2	phenanthrene	0.7±0.3	0.7±0.3	0.5±0.2
	3	dibenzofuran fluorene phenanthrene 1-methylphenanthrane fluoranthene pyrene	1.5±0.8	1.4±0.1	1.0±0.5

[a] The mean and standard deviation for controls was: 0.531±0.213 based on 8 samples, each containing 15, 6-10-week-old fathead minnows.

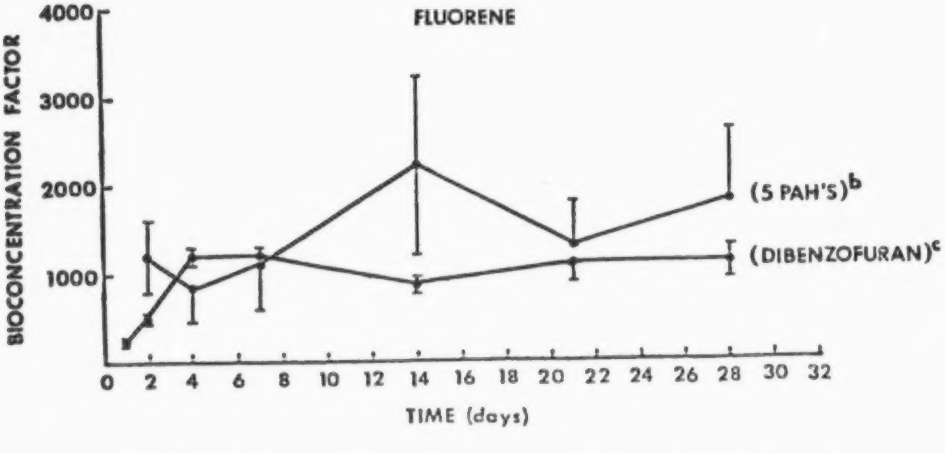
[b] The AHH values in this experiment were based on duplicate determinations, whereas all others were done in triplicate.

FIGURE 10
SUMMARY OF DIBENZOFURAN
EXPERIMENTS[a]



[a]Based on data in Table 15. [b]Exp. #3, Tank #3; fluorene, phenanthrene, 1-methylphenanthrene, fluoranthene, and pyrene also present. [c]Exp. #1, Tank #1; fluorene also present.

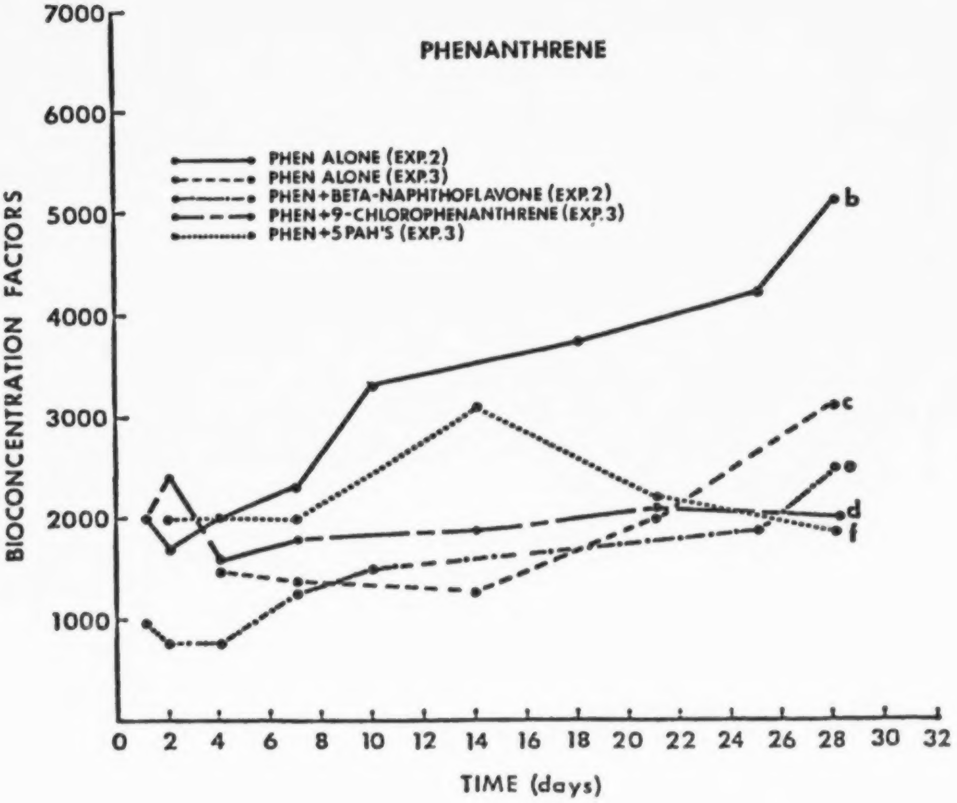
FIGURE 11
SUMMARY OF FLUORENE
EXPERIMENTS[a]



[a]Based on data in Table 15. [b]Exp. #3, Tank #3; dibenzofuran, phenanthrene, 1-methylphenanthrene, fluoranthene, and pyrene also present. [c]Exp. #1, Tank #1; dibenzofuran also present.

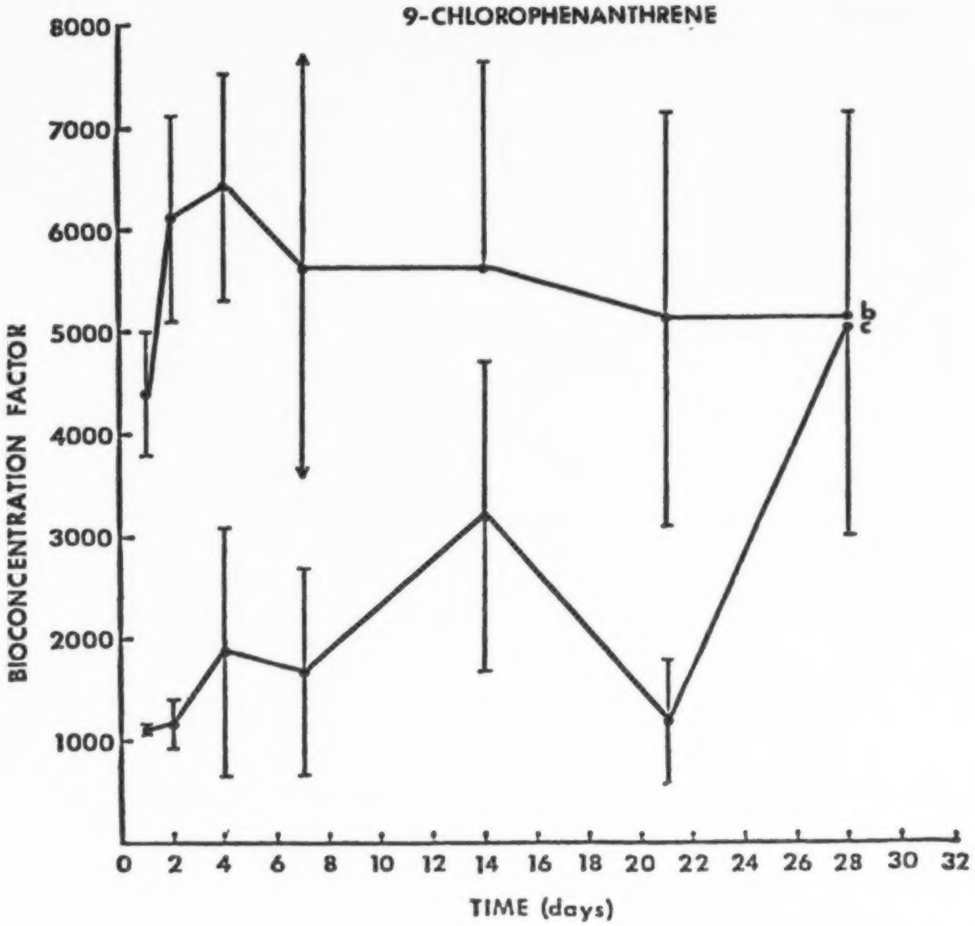
FIGURE 12

SUMMARY OF PHENANTHRENE EXPERIMENTS[a]



[a] Based on data in Table 15. [b] Exp. #2; no other compounds present. [c] Exp. #3, Tank #2, no other compounds present. [d] Exp. #3, Tank #1; 9-chlorophenanthrene also present. [e] Exp. #2, Tank #1; B-naphthoflavone also present. [f] Exp. #3, Tank #3; dibenzofuran, fluorene, 1-methylphenanthrene, fluoranthene, and pyrene also present.

FIGURE 13
SUMMARY OF 9-CHLOROPHENANTHRENE EXPERIMENTS[a]

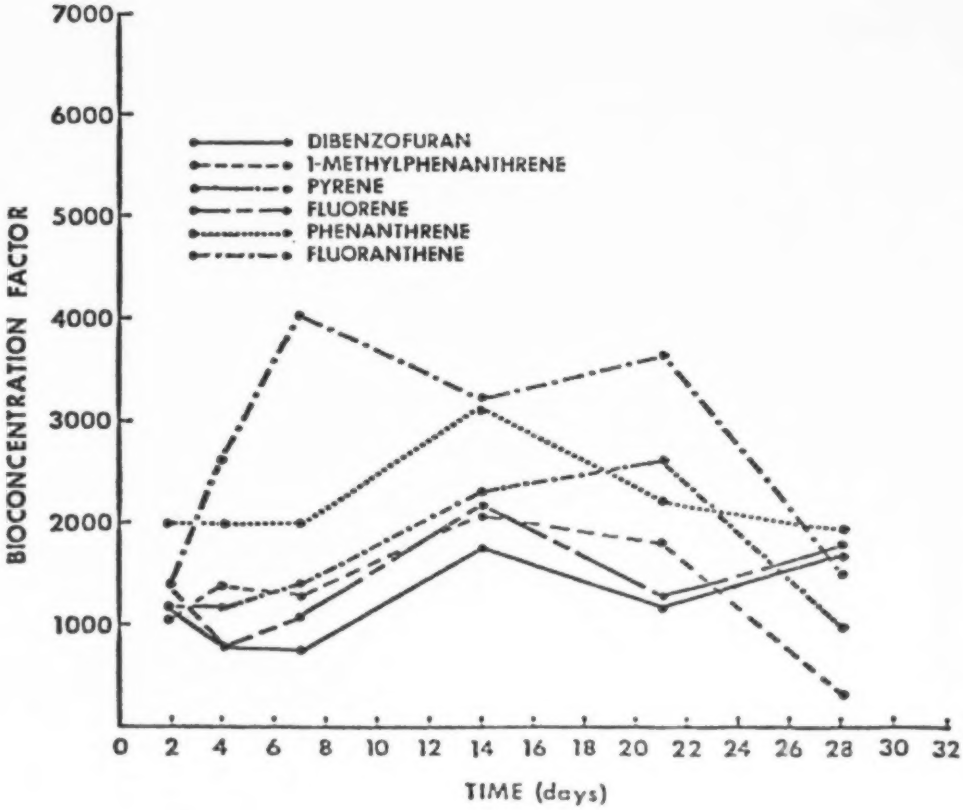


[a] Based on data in Table 15. [b] Exp. #3, Tank #1; phenanthrene also present. [c] Exp. #1, Tank #2; no other compounds present.

FIGURE 14

SUMMARY OF EXPERIMENT

WITH SIX PAH COMPOUNDS[a]



[a]Table 15, Exp. #3, Tank #3.

extracted with a Soxhlet apparatus with acetonitrile. Analysis of the extract by HPLC indicated that less than 1% of the compounds found in the precolumn (i.e., dissolved in 2.5 ℓ of water) was in the extract (i.e., associated with the particulates in 2.5 ℓ of water).

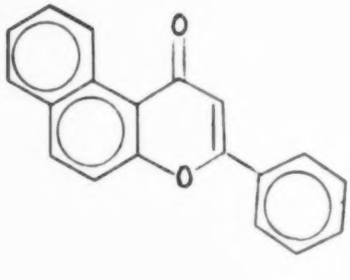
Results and Discussion

The results of these experiments are summarized in Tables 15-18 and Figures 8-14.

The reproducibility within experimental error of bioconcentration factors for phenanthrene in the absence of other compounds (Table 15: exp. #2, tank #2 and exp. #3, tank #2) and for all compounds of experiment #3 on day 28 (Table 15: exp. #3 all tanks, day 28, trials #1,2) as well as the similar lipid content of the fish indicate that the data presented in Table 15 can be utilized for trend analysis. The moderately large errors associated with the calculated bioconcentration factors (Table 15) are mainly the result of variations in the concentration of the compounds in the water (Table 17). Such variations are common in experiments of this type[221].

Bioconcentration factors approached a limiting value in most experiments. However, there are some clear exceptions in which the bioconcentration factors rose to a maximum value and then declined (experiment #3, tank 3). In this latter experiment it is possible that increased aryl hydrocarbon hydroxylase (AHH) enzyme activity (Table 18) may have been involved in the diminution of bioconcentration factor values for phenanthrene, 1-methylphenanthrene, fluoranthene, and pyrene in the second half of the experiment (Table 15; experiment #3, tank #3 and Figures 12,14).

Further statements about the relationship between AHH activity and bioaccumulation are difficult to make. The rather large errors involved in the AHH activity measurements using the reported fluorimetric procedure[198] and the previously mentioned errors in bioconcentration factor values make conclusions tenuous. For example, the presence of β-napthoflavone



(5,6-benzoflavone), which has been reported to be an AHH inducer[210.212,243], showed little significant increase in AHH activity (Table 18, exp. #2). However the fish in the tank containing the flavone (Table 16, exp. #1) appeared to release or metabolize accumulated phenanthrene at a significantly faster rate than the fish in the tank containing only phenanthrene (Table 16, exp.#2).

In contrast to β-naphthoflavone, 9-chlorophenanthrene appeared to show a significant increase in AHH activity (Table 18, exp. #1,3) but bioconcentration factors for phenanthrene in the presence or absence of this chloro compound were the same, within experimental error (Table 15, exp. #3, Tanks #1,2). Depuration rates in this latter experiment were also nearly identical (Table 16, exp. #3, Tank #1,2).

To summarize, the PAH's studied exhibited significant bioconcentration factors (1000-5000) but the attainment of a steady state can not always be assumed due to the presence of other compounds in the water or tissue or the level of mixed-function oxidase activity in the exposed fish. The release or metabolism of bioaccumulated PAH was usually rapid (< 4 days) after the fish were returned to relatively PAH-free water.

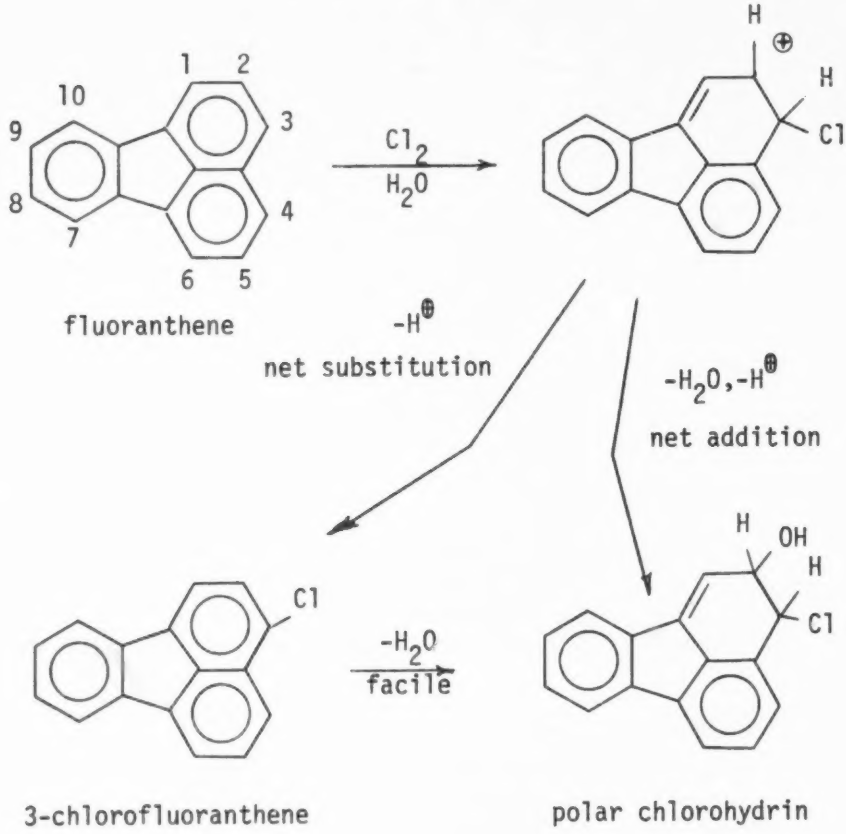
AQUEOUS CHLORINATION OF POLYNUCLEAR AROMATIC HYDROCARBONS

Chlorination is the predominant technique used for water renovation and disinfection. The process has been applied to wastewater treatment problems, to the disinfection of drinking water and the preservation of acceptable water quality through distribution systems, to the solubilization of sludge (a "superchlorination" process involving large doses of chlorine), to the maintenance of hygienic conditions in closed swimming areas, and to the reduction of algal and bacterial growth in cooling towers[260-265]. The development of the technology for the effective application of chlorine has been considered largely responsible for saving thousands of lives that could have been lost through contracting any of several possible water-borne diseases. In short, chlorination has developed into what has been referred to as the most valuable and versatile tool available to the water chemist[260,266].

The possible reaction of chlorine with materials present in the treated water has long been recognized[267], mainly because of the very practical necessity for using more chlorine than was anticipated to meet given standards of turbidity, BOD[267-269], or fecal coliform bacteria. Environmentally, chlorine and chloramines (as reaction products of ammonia, amino acids, or other amines with chlorine) are considered deleterious[264,270], and considerable effort has been directed toward their removal by such processes as reduction (e.g., SO_2) or by adsorption-decomposition (activated charcoal), with the result that documented examples of incorporation of carbon-bound chlorine under conditions used in water treatment have been quite limited. Early chemical investigations were only initiated when the chlorination process generated problems of taste and odor[266,271-274]. However, since these initial reports, there have been documented examples of the incorporation of chlorine into such systems as "activated" aromatics, humic acids, and nucleic acids[275-280]. Typically, the chlorine incorporation into these systems results in decreased degradability and increased toxicity[266-269]. Similar studies involving the ubiquitous[126] and, in some cases, carcinogenic[53] polynuclear aromatic hydrocarbons (PAH's) have not been extensive[54]. However, it has been reported that the concentration of various PAH's in water is reduced upon chlorination[141, 281-293] and that a few of the resulting products have been identified: chlorinated naphthalene[291,293] and C_{2-3}-naphthalenes[283], chlorinated acenaphthalenes[293], 5-chloro-3,4-benzopyrene[292] and 3,4-benzopyrene-5,8-quinone[156,292] as well as arene oxides[290].

Results

The results of the application of the C-18/HPLC/GC-PID/GC-MS method to the study of aqueous chlorination reactions of PAH's are provided in Table 19. Chlorinated PAH standards that were prepared and used in this work are listed in Table 20. The study demonstrates the vulnerability of PAH's to conversion to "second-order" products during dilute aqueous chlorination conditions typical of those encountered during disinfection processes. Moreover, the increasing reactivity of chlorine with decreasing pH is demonstrated in all examples examined. In the investigation fluoranthene showed the most interesting behavior of all the compounds studied. In one chlorination experiment it produced a very polar compound which eluted from the reverse-phase HPLC column in approximately ten minutes less than the time required for the monochlorofluoranthene standard. However, the GC retention times were identical and the GC-MS of the product indicated it to be a monochlorofluoranthene. Presumably, this product is a chlorohydrin which readily loses water to form a monochloro derivative. In a second experiment under similar conditions, the elimination of water apparently occurred before the HPLC analysis, since the product had an HPLC retention time identical to the monochlorofluoranthene standard. In contrast to the other compounds studied, experiments at pH ∿4 with phenanthrene and its 1-methyl derivative gave very low total recoveries for products (∿40% and ∿8% respectively). Presumably very polar products were generated in these reactions which were not concentrated by the 7x50 mm C-18 column.



Product identification was accomplished by MS and NMR data and, where possible, by a preparative scale chlorination in acetic acid (see Table 20) for comparison with known samples or reported melting points. The MS data is most useful for determining chlorine content (e.g., monochloro, dichloro, ...etc.) but is less useful in assigning the position(s) of substitution. Representative MS data are included in Appendix C. This appendix also includes some incomplete work on the dibenzofuran system, where both monochloro and dichloro derivatives were prepared, and pyrene, where it appears that trichloropyrene is formed.

The structural assignments of the major PAH derivatives are sometimes tenuous. With this in mind, we thought it prudent to catalog wherever possible the ^{13}C NMR spectra of all our chlorinated products. The ^{13}C spectra (with simultaneous ^{1}H decoupling) is very sensitive to the substitution pattern (both in chemical shifts and relative intensities). Data of this type, hopefully, will eventually be used not only for the present structural assignments, but to help identify previously unreported chlorinated PAH's that no doubt will be detected in these or other studies. It is also possible that high resolution (100 MHz or greater) ^{1}H NMR spectra with appropriate spin decoupling experiments can be invaluable in certain structural assignments. We have used this approach successfully to help establish the monochlorination product of phenanthrene as 9-chlorophenanthrene. This was done by examination of the relative chemical shifts of the three de-shielded protons (at C-1, C-8, and C-10) upon saturation of the remaining six aromatic protons located further upfield. The ^{13}C spectral data as well as the ^{1}H 100 MHz data are given in Appendix D. For a more detailed interpretation of the fluorene system the reader is referred to the M.S. thesis of Kenneth Welch, "Coal Derived PAH's and their Aqueous Chlorination Chemistry", University of Minnesota, Duluth, 1979.

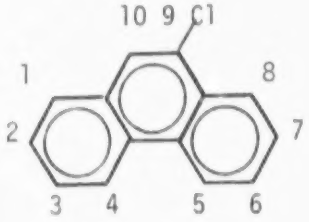


TABLE 19

Summary of Aqueous Chlorination Studies

Starting PAH	[Cl_2] mg/ℓ	[PAH] ng/ℓ	Reaction Time, hr	pH	Products Identified (% Yield)[d]	Basis for Assignment	Comments
1-Methylnaphthalene	24.0	531	3.0	3.8	Monochloro-1-methylnaphalene	a,b	M^+ = 176,178. GC retention time is identical to compound 1 (Table 20).
1-Methylnaphthalene	20.4	336	3.0	4.1	Monochloro-1-methylnaphthalene (73±5)	b,c	GC retention time identical to compound 1 (Table 20).
Fluorene	1.205	334	0.5	7.0	Fluorene (73±4)	b,c	
Fluorene	18.8	819	3.0	4.1	Fluorene	a,b,c	M^+ = 166
Fluorene	23.5	773	3.0	3.4	Fluorene Monochlorofluorene	a,b,c a	M^+ = 166 M^+ = 200, 202
Fluorene	21.3	333	3.0	3.35	Fluorene (~5) Monochlorofluorene (52±4)	b,c b,c	GC retention time is identical to compound 2 (Table 20).
Anthracene	12.9	1000	3.7	4.0	Anthracene (3) Anthraquinone (90± 11)	a,b,c a,b,c	M^+ = 178 M^+ = 208
Anthracene	0.0	1042	4.0	4.4	Anthracene	c	No anthraquinone produced according to HPLC
Anthracene	12.4	965	3.75	6.5	Anthraquinone (78±9)	b,c	
Anthracene	2.0	552	0.08	7.1	Anthraquinone (61±16)	b,c	
Phenanthrene	3.2	820	0.5	7.1	Phenanthrene	a,b,c	M^+ = 178.

Table 19 (Continued)

Starting PAH	[Cl_2] mg/ℓ	[PAH] ng/ℓ	Reaction Time, hr	pH	Products Identified (% Yield)[d]	Basis for Assignment	Comments
Phenanthrene	3.7	236	0.5	6.8	Phenanthrene (77±14)	b,c	
Phenanthrene	26.3	233	3.0	6.0	Phenanthrene (86±4)	b,c	
					Monochloro-phenanthrene (4±1)	b,c	Same GC retention time as compound 3 (Table 20).
Phenanthrene	19.3	820	3.0	4.1	Monochloro-phenanthrene	a,b	M^+ = 212, 214. Same GC retention time as compound 3 (Table 20).
Phenanthrene	19.5	239	3.0	4.2	Phenanthrene (9±4)	b,c	
					Monochloro-phenanthrene (38±5)	b,c	Same GC retention time as compound 3 (Table 20).
Phenanthrene	20.0	118	3.0	4.05	Monochloro-phenanthrene (39±5)	b,c	Same GC retention time as compound 3 (Table 20).
1-Methylphenanthrene	3.1	925	0.5	6.9	1-Methylphenanthrene	a,b,c	M^+ = 192.
1-Methylphenanthrene	21	994	3.0	4.0	Monochloro-1-methylphenanthrene	a,b	M^+ = 226,228. GC retention time identical to compound 5 (Table 20)
1-Methylphenanthrene	25.6	178	3.0	4.0	Monochloro-1-methylphenanthrene (~8)	b,c	GC retention time is identical to compound 5 (Table 20).
Fluoranthene	3.4	824	0.5	6.8	Fluoranthene	a,b,c	M^+ = 202
Fluoranthene	22.0	239	3.0	5.9	Fluoranthene (63±3)	b,c	

Table 19 (Continued)

Starting PAH	$[Cl_2]$ mg/ℓ	[PAH] ng/ℓ	Reaction Time, hr	pH	Products Identified (% Yield)[d]	Basis for Assignment	Comments
Fluoranthene	17.7	824	3.0	4.1	Fluoranthene	a,b,c	M^+ = 202
					Fluoranthene chlorohydrin	See comments.	Appears to lose water readily to form a monochloro fluoranthene. GC retention time identical to compound 6 (Table 20). M^+ = 235,238. However, reverse phase HPLC retention time was much less than compound 6 (Table 20).
Fluoranthene	23.9	239	3.0	4.03	Fluoranthene (42±3)	b,c	
					Monochloro-fluoranthene (32±1)	b,c	GC and HPLC retention times are identical to compound 6 (Table 20

Footnotes for Table 19:

[a] Mass spectral data. [b] GC retention time was identical to an authentic standard. [c] HPLC retention time was identical to an authentic standard. [d] Not corrected for recovery efficiency.

Table 20
Monochloro Products
Produced by Preparative Scale Chlorination Reactions in Acetic Acid[a]

PAH	Moles PAH: Moles Cl_2	Reaction Time, hr	Monochloro Products	Product Reference Number	Comments	Literature
1-Methylnaphthalene	1:3	18	Monochloro-1-methylnaphthalene	1	Oil M^+ = 176, 178	1-Chloro-4-methylnaphthalene (295,296)
Fluorene	1:1	3	Monochlorofluorene	2	Mp = 93 °C M^+ = 200,202	2-Chlorofluorene, mp 96.5° (297)
Phenanthrene	1:1	7.3	Monochlorophenanthrene	3	Mp = 52-52.5 °C M^+ = 212, 214	9-Chlorophenanthrene, mp 53-53.5° (298)
1-Methylphenanthrene	1:1	18	Monochloro-1-methylphenanthrene	4	Oil M^+ = 226, 228	
			Monochloro-1-methylphenanthrene	5	Mp = 87.5-88 °C M^+ = 226, 228	
Fluoranthene	1:2	18	Monochlorofluoranthene	6	Mp = 98-99 °C M^+ = 236, 238	3-chlorofluoranthene, mp 101-102° (299,300)

Footnotes for Table 20

[a] Temperature = 25 °C.

Experimental Details

Procedure for aqueous chlorination reactions[156]--

Purified water was placed into a pressure tank and, while stirring with an overhead stirring motor, a solution of sodium hypochlorite[260] was added, followed by sufficient 0.1 N sulfuric acid to attain the desired pH. The "free chlorine" concentration of the reaction mixture was determined iodometrically.[260,294] An acetonitrile solution of the PAH was then added via a 100 μl syringe. After the desired reaction time, the reaction was quenched by addition of solid sodium thiosulfate (twice the number of chlorine equivalents). The tank was then fitted with a 7x50 mm C-18 column and the quantitative analysis proceeded as described in Section 5. For GC-MS identification work it was necessary to remove the water from the individual HPLC fractions and to concentrate them. The water removal was effected by addition of ∿0.5 ml of methylene chloride to cause separation of layers, followed by removal of the aqueous layer with a Pasteur pipette. The organic layer which remained was dried with sodium sulfate and concentrated under a stream of nitrogen.

Procedure for preparative-scale chlorination reaction--[156]

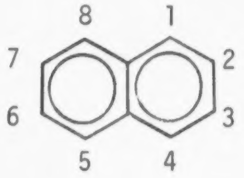
Chlorinated PAH's required for comparison with the products of the aqueous-chlorination reactions were prepared by the reaction of the parent PAH with chlorine gas in acetic acid[295]. Work-up of these reactions consisted of dilution with water followed by washing with sodium bicarbonate, drying with magnesium sulfate, and solvent evaporation. The material that remained was then separated and/or purified by preparative-scale HPLC using two 7x600 mm columns packed with either Bondapak C-18 Porasil^{R}B (37-75 μ, reverse phase) or Porasil^{R}A (37-75 μ, normal phase). The compounds obtained were recrystallized and checked for purity by GC-MS (see Table 20 and Appendix C).

SECTION 7

SYNTHESIS OF METHYLATED NAPHTHALENES

Among the ubiquitous PAH's in the aqueous environment, a large number of methylated derivatives are included[301]. The monitoring of these potentially hazardous compounds have been described by Dr. F.C. Monastero of the U.S. Department of the Interior as "a national imperative"[302]. The monitoring of even the methylated PAH's is no easy task owing to the complexity of the existing mixtures and the lack of suitable standards. The need to develop synthetic routes to methylated PAH's is summarized in a recent report by Dr. J.E. Tomaszewski of the Chemical Carcinogenisis Section of the NCI Frederick Research Center[303].

The magnitude of the complexity of this problem can be gained by examination of the simplest PAH, naphthalene. There are 73 methylated naphthalene derivatives with one to eight methyl groups substituted at the eight available positions. Of these, only seven are presently commercially available.



The present work describes a synthetic design that leads to the selective synthesis of certain mono-, di-, tri-, tetra-, and pentamethyl naphthalenes. The synthetic scheme appears to be applicable to the anthracene and penanthrene nuclei as well.

The new procedure is based on the susceptibility of the readily available naphthalene oxide system 1 to ring opening reactions with alkyllithiums[304]. The general route is illustrated below for the synthesis of 2-methylnaphthalene (2). This particular naphthalene is commercially available,* but not in the 100% isomeric purity as produced in the scheme.

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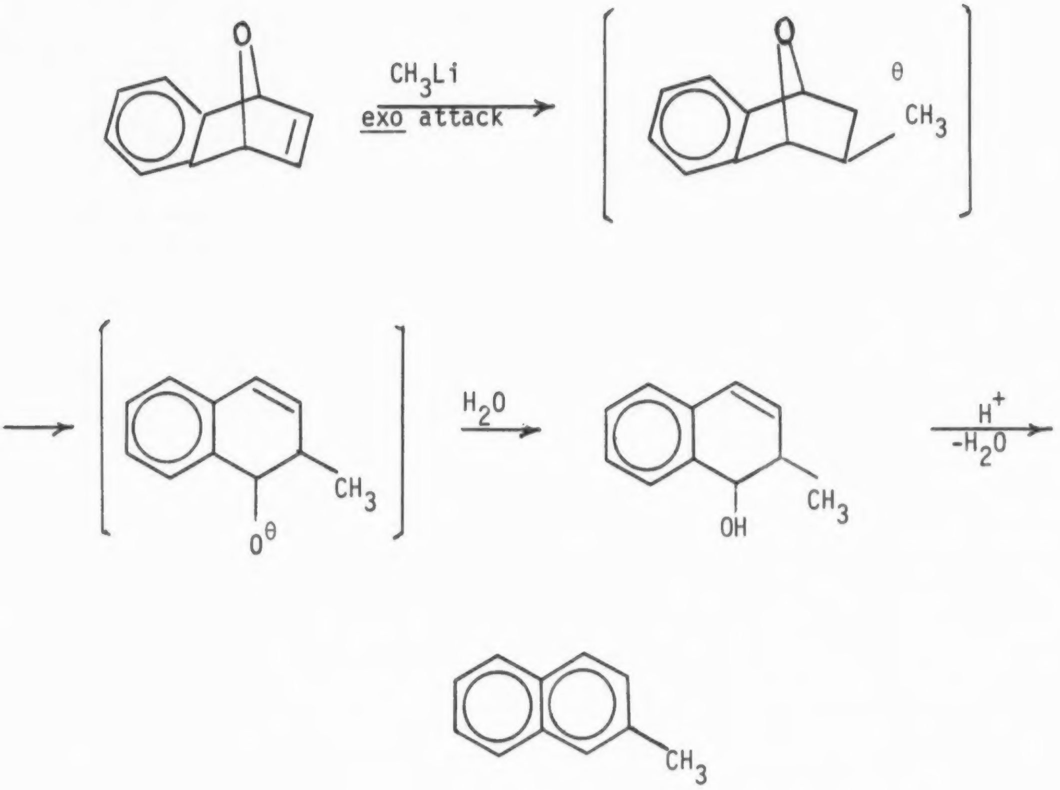
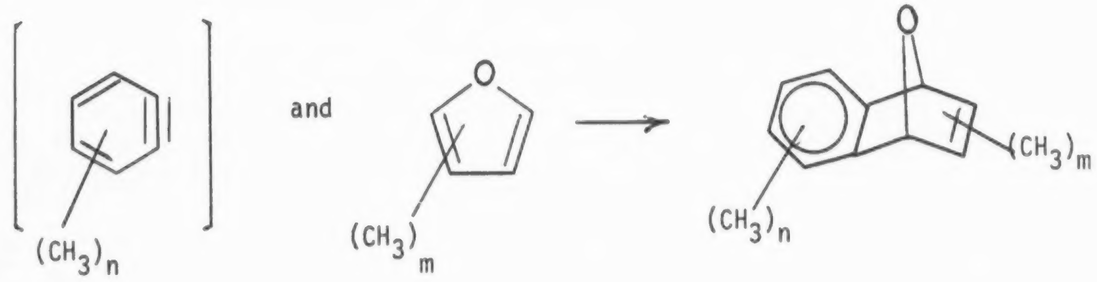
* Aldrich Chemical Company, Milwaukee, Wisconsin


O
CH3Li
exo attack
θ
CH3
Oθ
H2O
OH
H+
-H2O
CH3

3

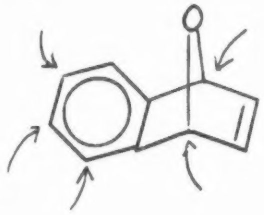
The value of this route is the flexibility that can be gained by introducing methyls in the component parts of the epoxynaphthalene synthesis[305].


(CH3)n
and
O
(CH3)m
(CH3)n
(CH3)m

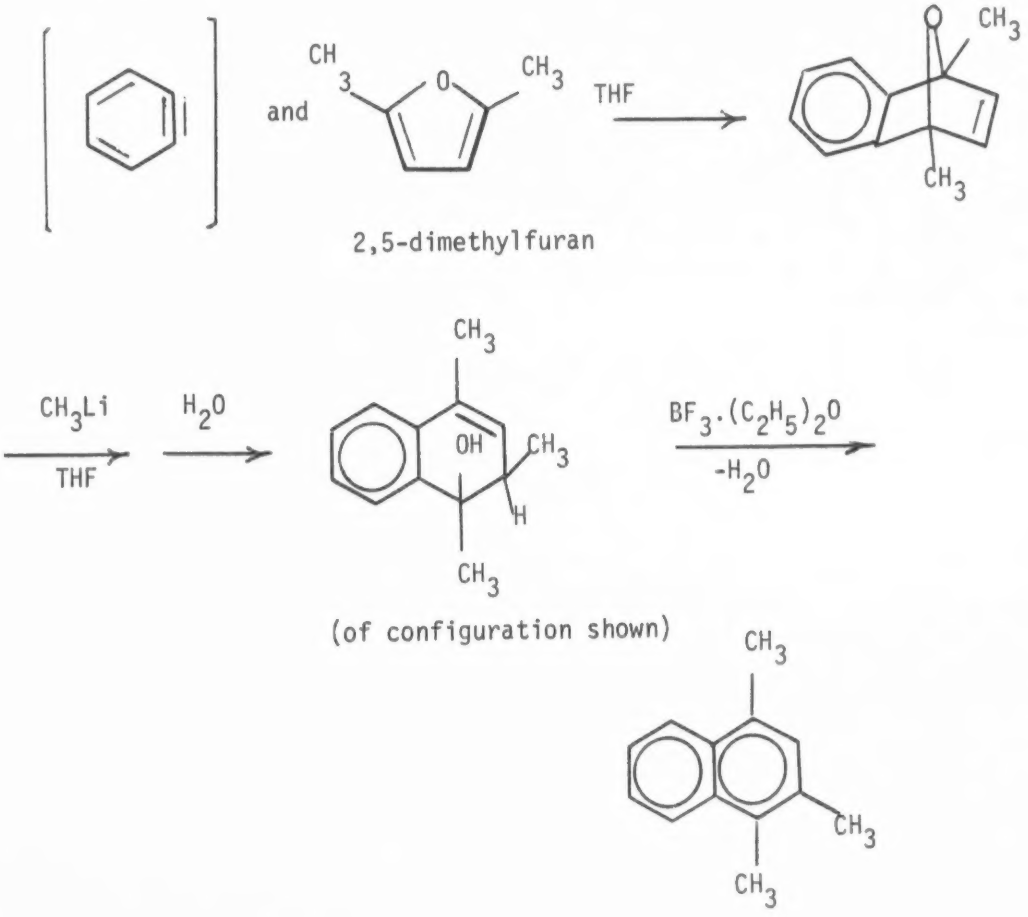
methyl benzynes
(generated *in situ*)

methyl furans

We have developed the procedure to the point where up four methyls may be introduced on the epoxynaphthalene systems at those positions indicated by the arrows.

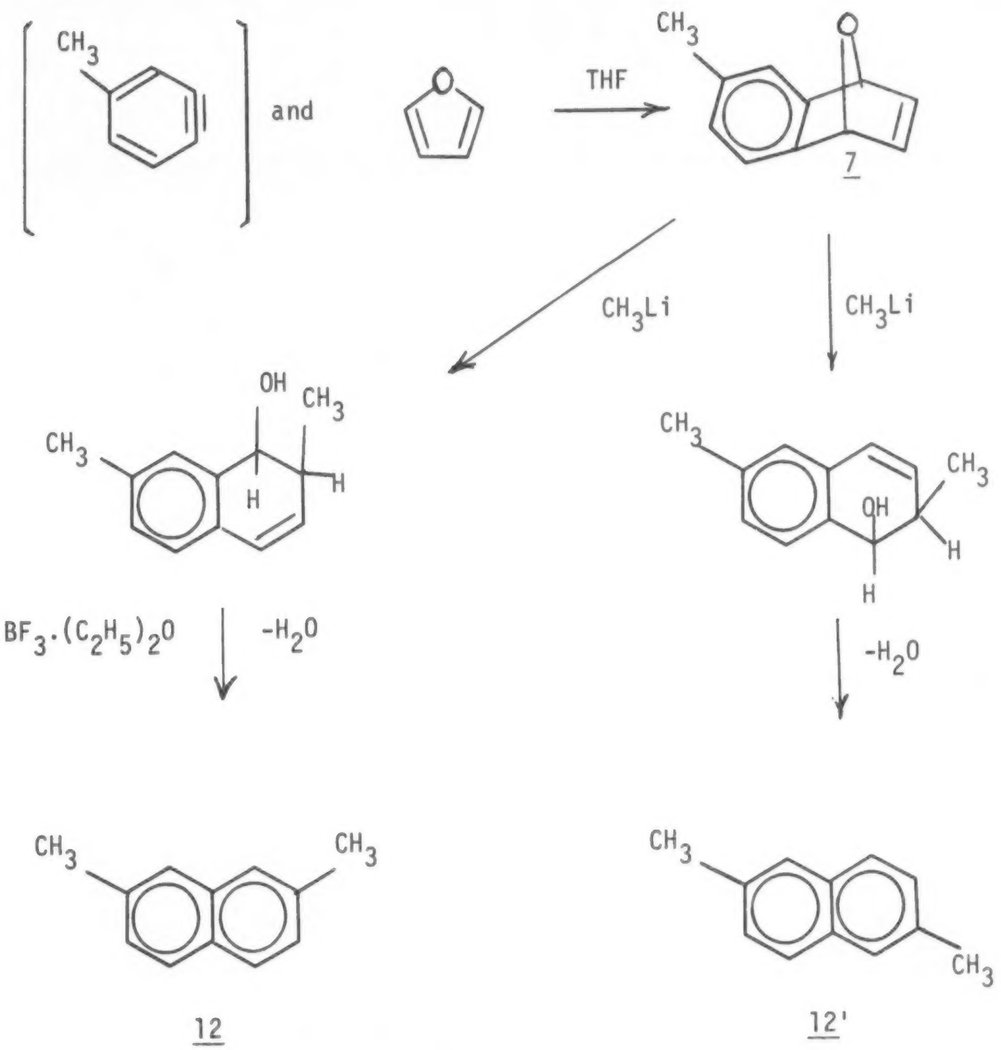


The reaction is illustrated below for the selective synthesis of 1,2,4-trimethylnaphthalene.


CH3
and
O
CH3
THF
CH3
CH3
2,5-dimethylfuran
CH3Li
THF
H2O
CH3
OH
CH3
H
CH3
(of configuration shown)
BF3·(C2H5)2O
-H2O
CH3
CH3
CH3

9

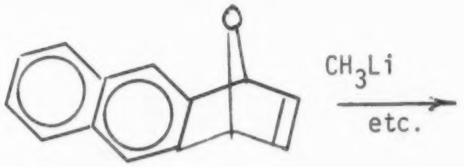
The only real synthetic limitation to this route, in addition to the availability of suitably substituted benzynes and furans, is the production of 2 isomeric products with the attack of methyllithium on an unsymmetrical epoxynaphthalene. This is illustrated below for the production of 2,6- and 2,7-dimethylnaphthalenes. This offers a limited separation problem for the isolation of pure individual isomers but nevertheless is amenable to a GC/MS analysis.

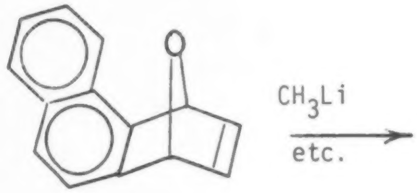

CH3
and
THF
CH3
7
CH3Li
CH3Li
OH
CH3
CH3
H
H
CH3
CH3
OH
H
H
BF3.(C2H5)2O
-H2O
-H2O
CH3
CH3
CH3
CH3
12
12'

Results

The synthetic scheme discussed above has been applied to the syntheses of the methylepoxynaphthalenes listed in Table 21. The resulting methylnaphthalenes resulting from the interaction with methyllithium are listed in

Table 22. The structures were confirmed by microanalyses, NMR (60 MHZ), IR, and MS techniques. Pertinent NMR data are listed in Appendix E and representative MS spectral data in Appendix F. The general scheme discussed here offers a potential route to certain methylanthracenes and phenanthrenes via the corresponding epoxyanthracenes and epoxyphenanthrenes.





Mass Spectra--

Mass spectra of each of these compounds have been obtained and presented in Appendix F. It should be noted that the mass spectra were run on mixtures of isomeric products, except in (3) and (9) which are isomer free products. The diagrams in Appendix F are labeled according to the major product.

Analysis of the mass spectra of these methylated compounds reveals two possible useful fragmentation trends. As the methyl substitution increases:

(1) The M^+/M^+-1 ratio increases, and
(2) The size of the M^+-15 (loss of a methyl radical from the radical cation) peak increases.

The radical cation may lose either a methyl radical or the elements of C_2H_2 or C_3H_4. A hydrogen atom may also be lost by the M^+ radical cation. With increasing methylation the loss of C_2H_2 or C_3H_4 becomes less pronounced.

Experimental Details

Apparatus--

The 60 MHz NMR spectra were obtained on a Varian EM-360 NMR instrument with tetramethylsilane used as an internal standard and deuteriochloroform as the solvent. Mass spectral data were obtained at 70 ev on a Varian CH-5 system by Mr. Douglas Kuehl of the EPA Environmental Research Laboratory, Duluth.

Microanalyses--

The microanalytical data were obtained by Spang Microanalytical Laboratory, Ann Arbor, Michigan. See Appendix G.

Reagents--

Furan and anthranilic acid were obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. The 3-methyl and 5-methylanthranilic acids were purchased from Pfaltz and Bauer, Inc., Stamford, Connecticut. Methyllithium was obtained from Alfa Inorganics, Inc. The 2,5-dimethylfuran was synthesized according to the procedure of Newman[306].

General Procedure for the Conversion of the Anthranilic Acids to the 1,2-dihydro- and 1,4-dimethyl-1,4-epoxynaphthalenes--

In a 250-ml 3-neck round-bottom flask equipped with a reflux condenser, magnetic stirrer, and pressure equilibrated dropping funnel were placed 30 m of 1,2-dimethoxyethane, 3 mℓ of isoamyl nitrate (0.022 mol) and 5 mℓ of furan (0.070 mol) or 2.5 dimethyl furan (0.045 mol)[6]. The reaction mixture was heated to reflux (furan, 80°, 2.5 dimethyl furan, 106°) under a nitrogen atmosphere. The appropriate anthranilic acid (ca. 0.014 mol) in 30 mℓ of 1,2 dimethoxyethane was added dropwise during 2½ to 3 hours. Following 30 min at reflux the reaction mixture was cooled, made basic with 10% sodium bicarbonate, and extracted with six equal portions of ether and water. The aqueous layer was re-extracted six times and the combined ether extracts were dried and decolorized. A short column distillation (1 mm, 70°) produced a pure sample.

The average yield for this conversion is 40%. MNR data were correct for the proposed structures. Microanalytical data are listed in Appendix G.

General Procedure for the ADdition of Methyllithium to the 1,4-Epoxynaphthalenes--

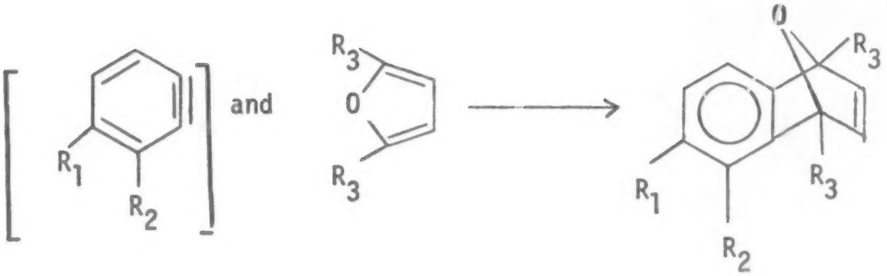
The epoxynaphthalene (1, 4, 5, 6, 7, or 8), 0.0039 mol, was dissolved in 50 mℓ of dry THF under an anhydrous nitrogen atmosphere in a 250-mℓ 3-nick round-bottom flask fitted with a reflux condenser and magnetic stirrer. Methyllithium, 0.045 mol, was added dropwise and the solution was refluxed for 90 min. The reaction mixture was then cooled to room temperature and water was added to destroy the excess methyllithium. The aqueous layer was extracted with six 25-mℓ portions of ether and the combined extracts were dried over anhydrous magnesium sulfate. A short path distillation (0.6 mm) produced a relatively pure alcohol (some thermal dehydration inevitably occurred) at a typical yield of about 40%. The alcohols were not purified further but carried directly to the dehydration step.

General Procedure for the Dehydration and Production of the Methylnaphthalenes--

The partially purified alcohols produced above were dissolved in about 50 mℓ of anhydrous ethyl ether and a few drops of freshly distilled borontrifluoride ether were added. The solution was stirred at room temperature for no more than 15 min. The ether was washed twice with 10-mℓ portions of 10% sodium bicarbonate. The ether layer was dried over anhydrous magnesium sulfate and, after removal of the solvent, the naphthalene was purified by a short path distillation (1mm,70°). A pure product in almost a quantitative yield was obtained in this step. The significant NMR data are in Appendix E, mass spectral data in Appendix F, and microanalytical data in Appendix G.

TABLE 21

SYNTHESIS OF POLYMETHYLEPOXYNAPHTHALENES


R1
R2
and
R3
O
R3
O
R3
R3
R1
R2

1, $R_1 = H$, $R_2 = H$, $R_3 = H$

4, $R_1 = H$, $R_2 = H$, $R_3 = CH_3$

5, $R_1 = H$, $R_2 = CH_3$, $R_3 = H$

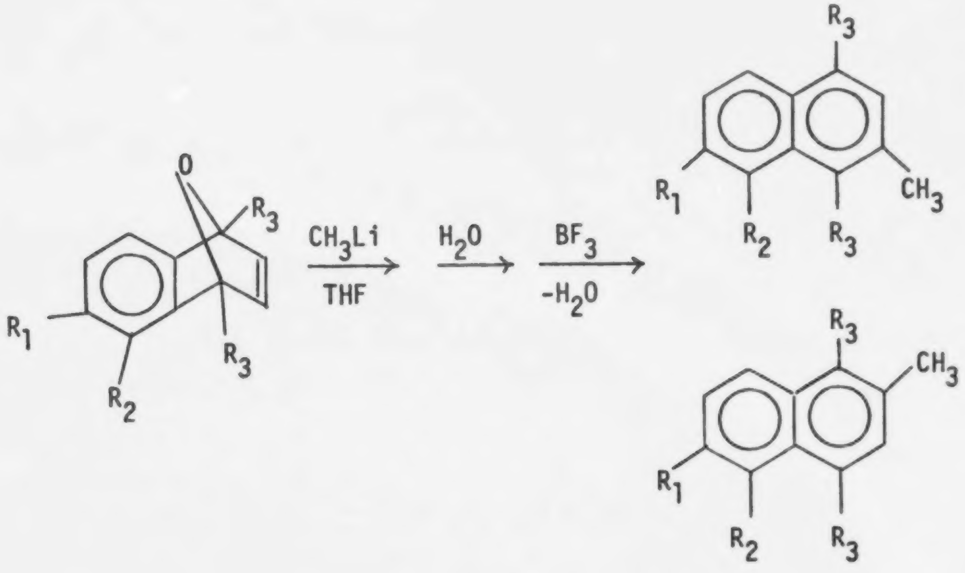
6, $R_1 = H$, $R_2 = CH_3$, $R_3 = CH_3$

7, $R_1 = CH_3$, $R_2 = H$, $R_3 = H$

8, $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$

TABLE 22

SYNTHESIS OF POLYMETHYLNAPHTHALENES


O
R_3
R_1
R_2
R_3
CH_3Li
THF
H_2O
BF_3
$-H_2O$
R_3
R_1
R_2
R_3
CH_3
R_3
CH_3
R_1
R_2
R_3

3, R_1 = H, R_2 = H, R_3 = H

9, R_1 = H, R_2 = H, R_3 = CH_3

10 and 10', R_1 = H, R_2 = CH_3, R_3 = H

11 and 11', R_1 = H, R_2 = CH_3, R_3 = CH_3

12 and 12', R_1 = CH_3, R_2 = H, R_3 = H

13 and 13', R_1 = CH_3, R_2 = H, R_3 = H

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APPENDIX A

ADDITIONAL TABLES AND FIGURES RELEVANT TO BIOLOGICAL STUDIES ON THE LEACHING AND VOLATILIZATION OF COAL

TABLE A-1

PROPERTIES OF COAL LEACHATE

	Distilled Deionized Water (7 samples)		Distilled Deionized Water Leachate (48 samples)		Lake Superior Water (48 samples)		Lake Superior Water Leachate (6 samples)	
	mean	range	mean	range	mean	range	mean	range
Dissolved Oxygen[a] (mg/l)	-	-	6.81	3.70-8.50	8.06	6.87-8.60	-	-
Total Alkalinity[b] (mg/l $CaCo_3$)	1.27	1.01-1.52	5.11	3.40-6.70	42.62	41.20-44.60	21.53	17.78-24.75
EDTA Hardness[b] (mg/l $CaCo_3$)	0.0	-	0.84	0.33-1.60	45.50	44.46-46.43	13.98	2.74-14.41
pH (20-22^{0}C)	-	5.55-6.92	7.8[c]	6.8-8.2	7.8[c]	7.5-7.9	7.8[c]	7.22-8.09
Conductivity (µmhos/cm)	1.19	0.73-1.63	27	15-32	92	90-104	70	65-77
Turbidity (N.T.U.)	-	-	4.45	1.1-8.8	0.54	0.16-1.9	2.4	1.3 - 3.2

[a] Reference R-294 azide modification

[b] Reference R-294

[c] Mode of pH values

TABLE A-2

EFFECTS OF COAL DISTILLATE ON *DAPHNIA PULICARIA*[a]

Coal Distillate Concentration (%)[b]	Conductivity (μmhos/cm)	Mortality (%)	Distilled Water Concentration (%)[b]	Conductivity (μmhos/cm)	Mortality (%)
0	88	0	10	82	0
20	72±6	1.7	25	70	0
40	56±1	3.3	50	48	0
60	42±2	10.0	75	27	0
80	24±1	58.3	80	19±1	83.3
			100	4.7	100

[a] *Daphnia* were exposed to coal distillate (and 80% distilled water) during 3 separate bioassays with 4 replicates per concentration. The conductivity of the exposure water was measured at the beginning of each bioassay and the mean and standard deviation indicated. The distilled water and lake water controls were tested in 1 bioassay, with 4 replicates per concentration.

[b] Coal distillate and distilled water were diluted with Lake Superior water.

TABLE A-3. FATHEAD MINNOW BEHAVIORAL RESPONSE TO COAL LEACHATE[a]

EXPOSURE	OBSERVATION OF SPAWNING COLORATION (week #1)	# SPAWNINGS	EGGS/SPAWNING (ave. #1)	FISH/TANK
Leachate (Tank 1)	-	-	-	19
Leachate (Tank 2)	23	-	-	8
Control (Tank 3)	15	-	-	18
Control (Tank 4)	16	5	43.6	18

[a] During a 24 week exposure to coal leachate, the onset of male spawning coloration was noted. Minnows were 2 mo. old at the start of the experiment. Tank 1 and 2 were leachate exposures and tanks 3 and 4 Lake Superior water exposures.

TABLE A-4

EFFECTS OF COAL LEACHATE ON SPAWNING SUCCESS IN FATHEAD MINNOWS

EXPOSURE Water	 g COAL/l	CONDUCTIVITY µmohs/cm	#WEEKS/TEST	#TANKS	#TANKS IN WHICH SPAWNING OCCURRED	# SPAWNINGS	MEAN # EGGS/ SPAWNING	HATCHABILITY, % (# EGGS)
100% Leachate	6.25	70 (65-77)	4	4	0	0	0	0
100% Leachate	6.25	70 (65-75)	2	3	1	2	85	88 (50)
90% Leachate	5.0	71 (66-78)	2	2	1	3	79	
75% Leachate	4.17	76 (71-83)	2	3	1	2	162	94 (100)
50% Leachate	2.78	82 (78-86)	2	2	1	3	170	88 (150)
25% Leachate	1.39	87 (85-89)	2	3	2	5	69	64 (150)
10% Leachate	0.55	91	2	5	2	2	97	81 (100)
Lake Superior Water	0	93 (92-94)	2	4	3	7	102	90 (200)
Lake Superior Water	0	91 (89-92)	4	4	4	16	70	72 (500)
25% Distilled water in Lake Superior Water	0	73 (71-74)	2	2	2	6	138	69 (300)

TABLE A-5

COUGH RESPONSE DATA FOR TWO BLUEGILL SUNFISH EXPOSED TO COAL DISTILLATE

TIME FRAME	CONCENTRATION (%)		AVE. COUGHS/MIN.	
	Tank 1	Tank 2	Fish 1	Fish 2
0800 - 0820	0	0	0.55	0.55
0915 - 0935	1	5	1.10	1.00
1000 - 1020	5	20	1.35	1.05
1300 - 1320	5	20	1.55	0.80
1550 - 1610	5	20	0.50	0.40
0800 - 0820	5	20	0.45	0.50

TABLE A-6

EFFECT OF COAL DISTILLATE ON HEPATIC MIXED-FUNCTION OXIDASE PARAMETERS IN RAINBOW TROUT[a]

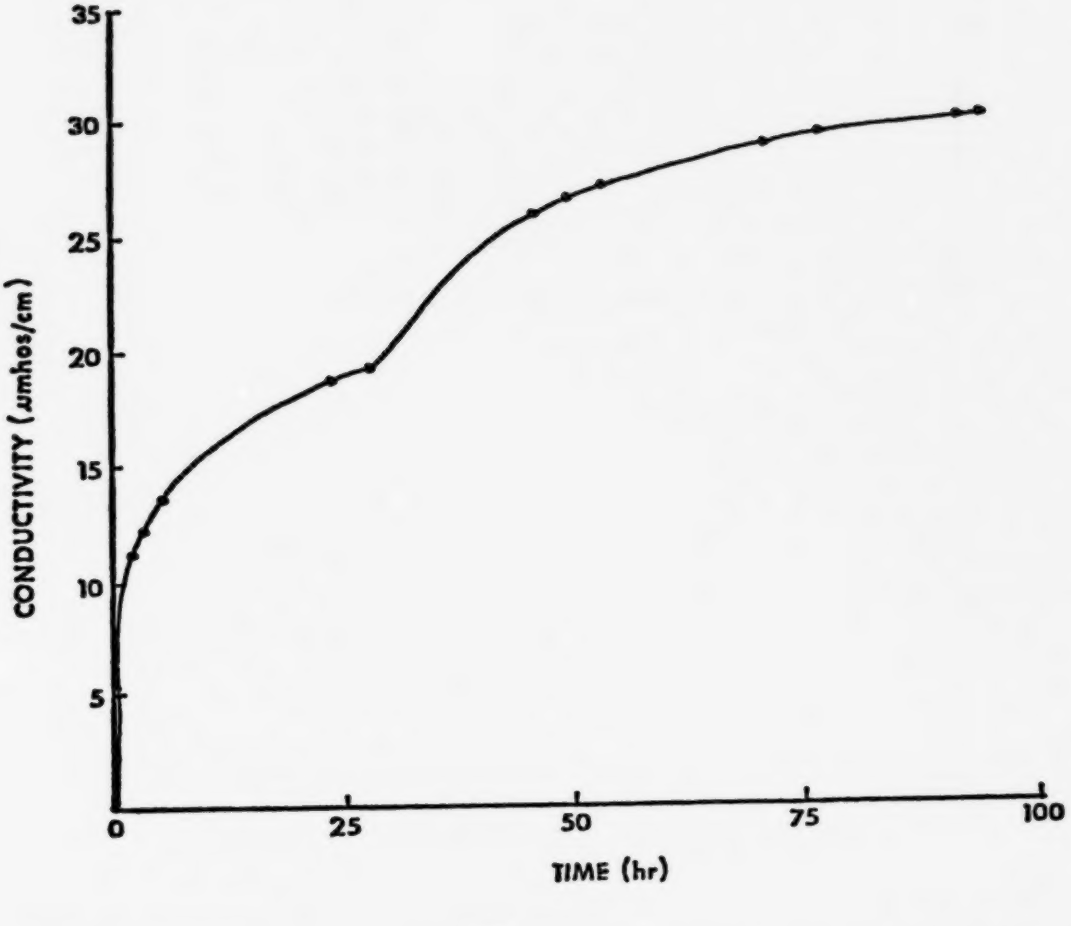
Day	P-450 (nM/mg protein)		AHH (pM/mg protein/min)	
	Control	Distillate	Control	Distillate
3	0.130	0.108	1.234 ±0.161	0.958 ±0.308
7	0.125	0.097	2.224 ±0.161	1.968 ±0.928
10	0.156	0.033	0.968 ±0.315	1.801 ±0.374
14	0.065	0.138	1.305 ±0.041	1.353 ±0.083
21	0.082	0.089	1.124 ±0.105	2.873[b] ±0.351
	0.112[c] ±0.037 (33%)	0.093[c] ±0.038 (41%)	1.371[c] ±0.494 (36%)	1.790[c] ±0.723 (40%)

[a] Rainbow trout were exposed to 0.2% coal distillate under flow-through conditions.

[b] Significantly elevated compared to controls.

[c] Mean and Standard deviation over the 21 day period.

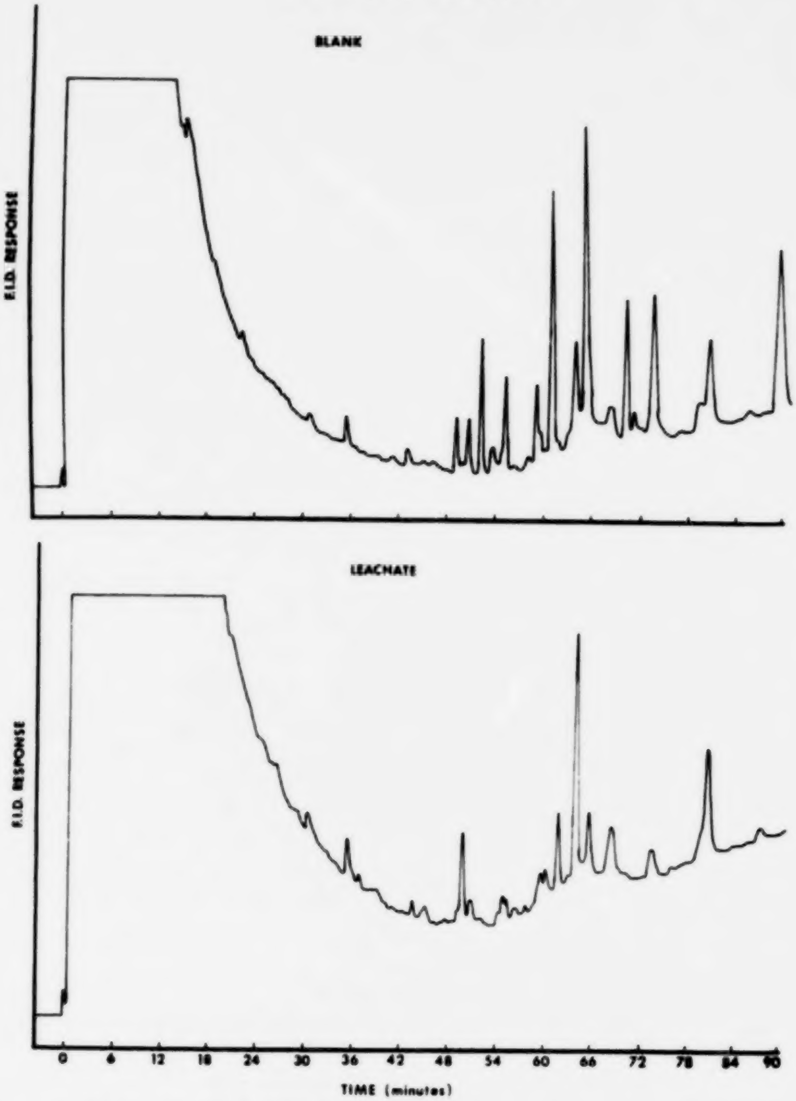
FIGURE A-1
CONDUCTIVITY CHANGES DURING LEACHING
OF COAL[a]



[a] Coal leaching was monitored after adding ground coal (< 0.5 mm) to distilled deionized water at a coal to water ratio of 6.3 g/l.

FIGURE A-2

GAS CHROMATOGRAPHIC ANALYSIS OF A HEXANE EXTRACTION OF COAL LEACHATE[a]

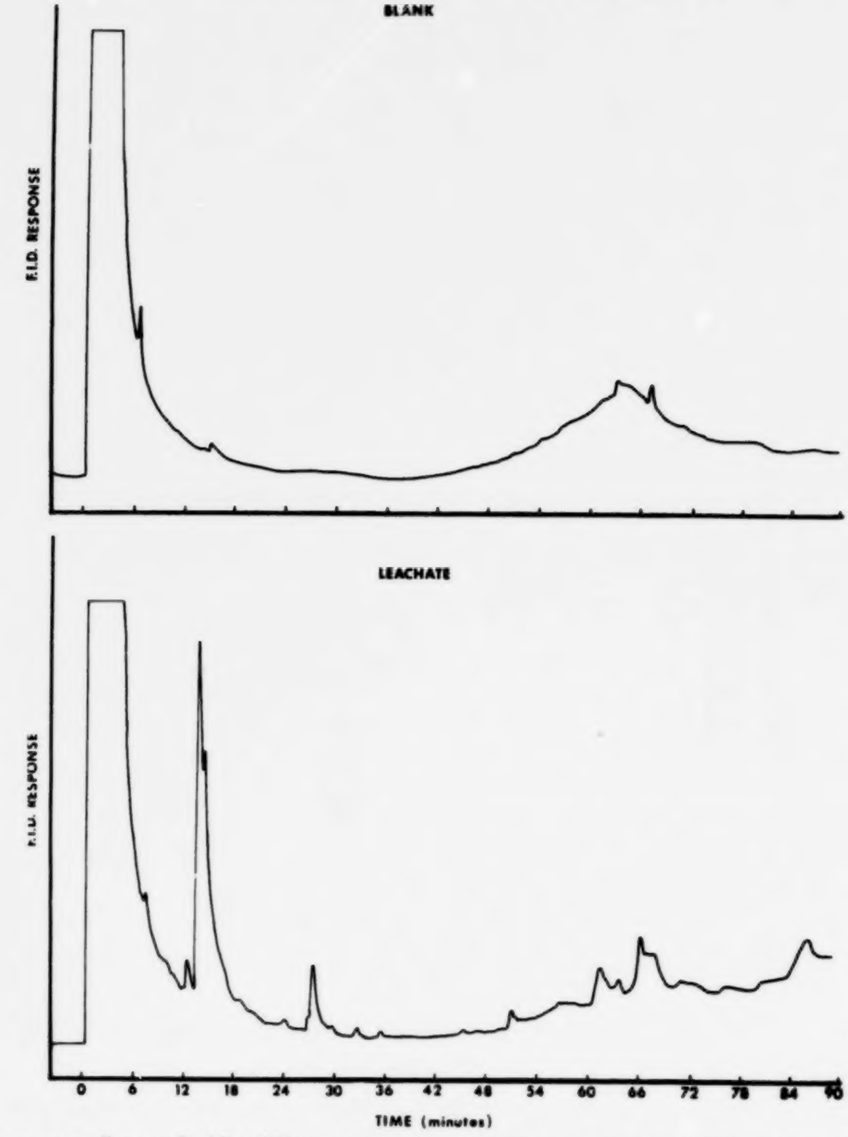


[a] Two liter samples of distilled deionized water and centrifuged coal leachate (6.3 g coal/L distilled deionized water) were extracted with 100 ml hexane.

GC conditions-column: 5% SP-2250, 2m x 2mm i.d. glass; injector: 250°C; detector: 300°C; carrier flow: 20 ml/min; program: 80-260°C at 4°/min.

FIGURE A-3

GAS CHROMATOGRAPHIC ANALYSIS OF A METHYLENE CHLORIDE EXTRACTION OF COAL LEACHATE[a]

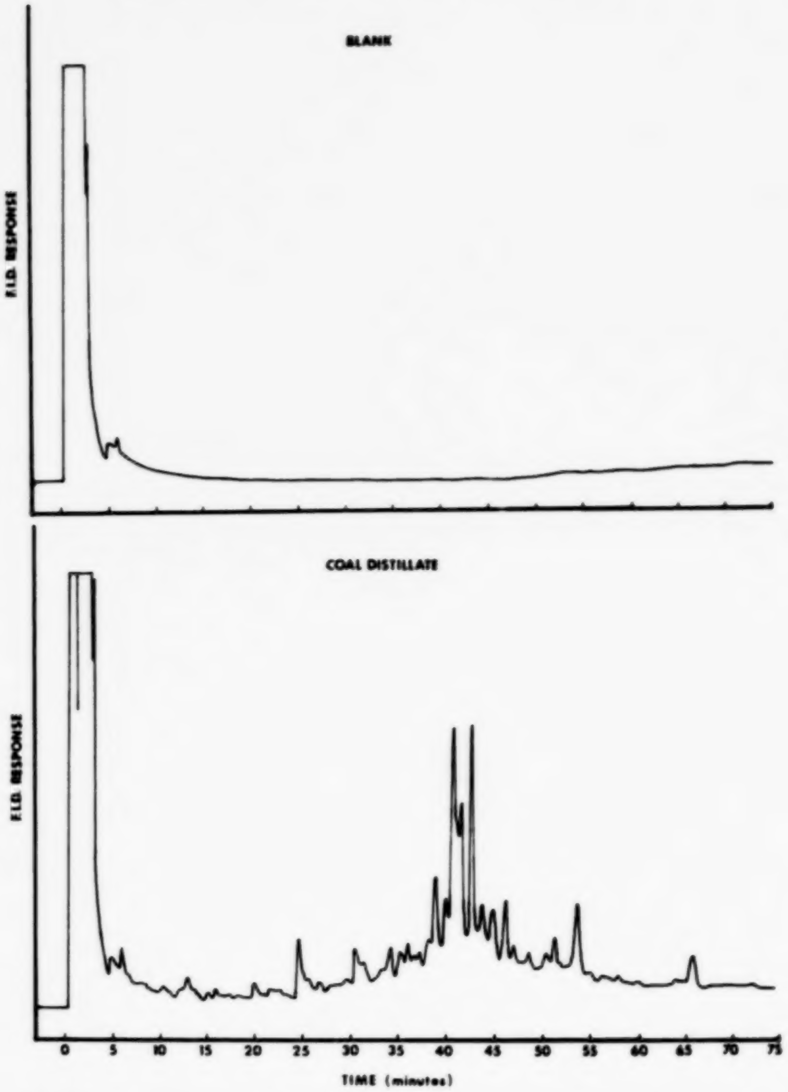


[a] One liter samples of distilled deionized water and centrifuged coal leachate (6.3 g coal/L distilled deionized water) were extracted with 50 ml methylene chloride.

GC conditions-column: 5% SP-2250, 2m x 2mm i.d. glass; injector: 250°C; detector: 300°C; carrier flow: 20 ml/min; program: 80-260°C at 4°/min.

FIGURE A-4

GAS CHROMATOGRAPHIC ANALYSIS OF AN
ISOOCTANE EXTRACT OF COAL DISTILLATE

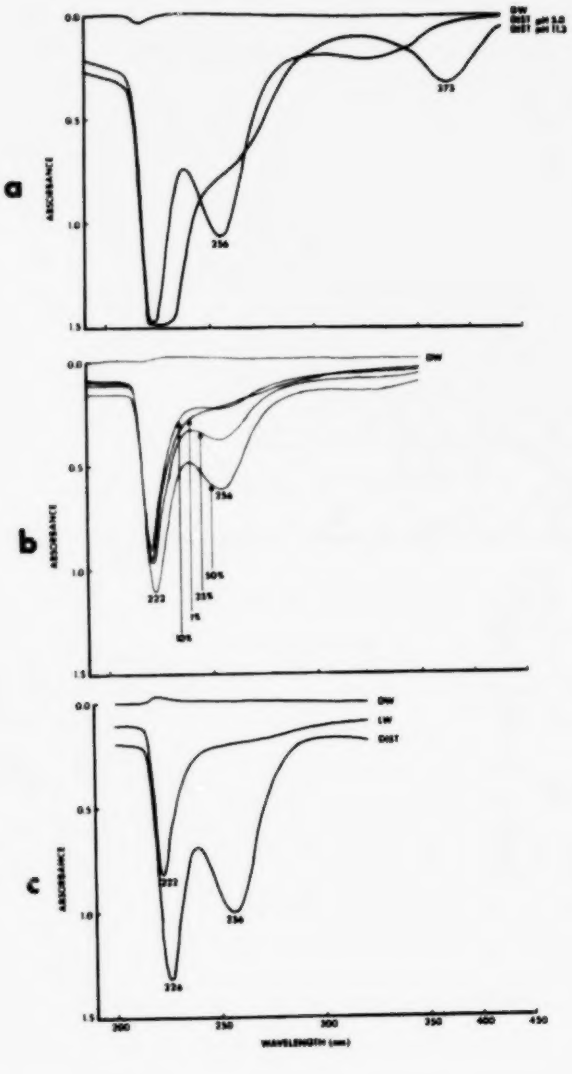


[a] A steam distillation of 1500 ml distilled deionized was carried out in a modified Nielsen-Kruger distillation apparatus for 6 hr.

[b] As above except that 100 g of coal (<0.250 mm) were added.

GC conditions-column: 5% SP-2250, 2m x 2mm i.d. glass; injector: 250°C; detector: 300°C; carrier flow: 20 ml/min; program: 80-250°C at 4°/min.

FIGURE A-5
UV SCANS OF COAL DISTILLATE



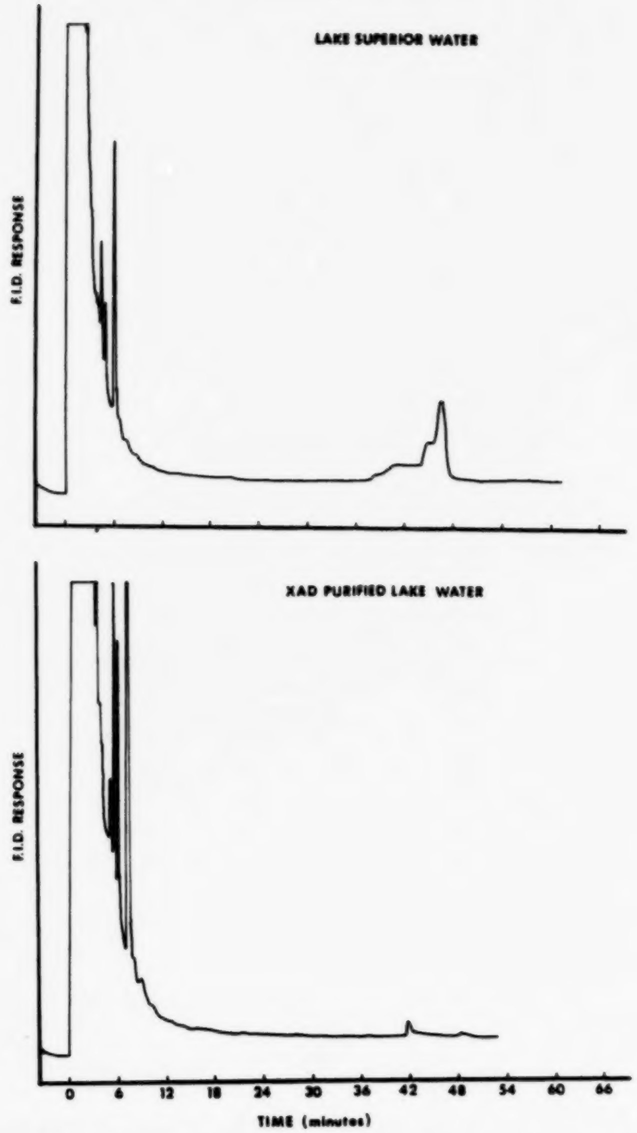
a. UV spectra of distilled water (DW), Lake Superior water (LW), and coal distillate (DIST).

b. After obtaining a stable baseline using distilled water, various dilutions of coal distillate were made using Lake Superior water as the diluent.

c. Coal distillate (pH 5.0) was scanned and then rescanned after raising the pH to 11.3 with 1 N NaOH.

FIGURE A-6

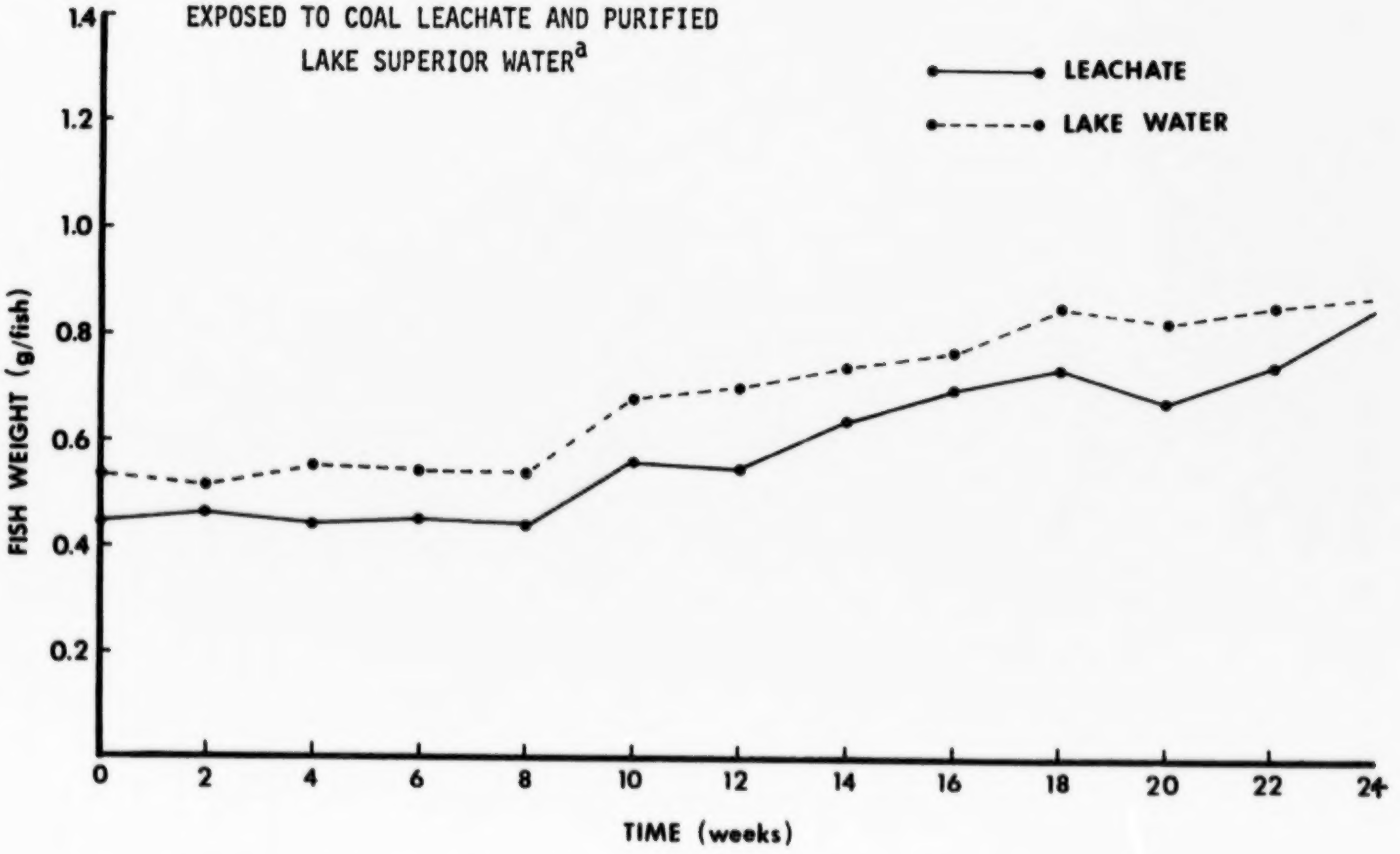
GAS CHROMATOGRAPHIC ANALYSIS OF A HEXANE EXTRACT OF XAD-2 PURIFIED LAKE SUPERIOR WATER[a]



[a] A one liter sample of Lake Superior water was collected before XAD treatment and another one liter sample taken after 13 liters of Lake Superior water had passed through the 50 ml XAD column. Concentrated hexane extracts were analyzed using the following GC conditions-column: 5% SP-2250, 2m x 2mm i.d. glass; injector: 250°C; detector: 300°C; carrier flow: 20 ml/min; program: 125°C 10 min, 125-200°C at 4°/min.

FIGURE A-7

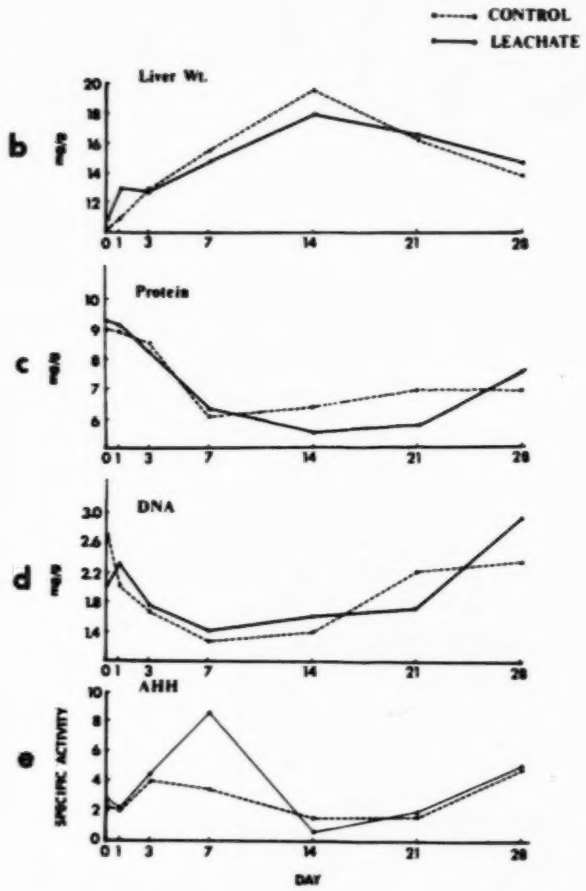
GROWTH RATES OF FATHEAD MINNOWS
EXPOSED TO COAL LEACHATE AND PURIFIED
LAKE SUPERIOR WATER[a]



[a] Fathead minnows, 2 months old initially, were exposed to coal leachate (6.3 g coal/L distilled deionized water) or to Lake Superior water (purified using XAD-2) under renewed static conditions.

FIGURE A-8

EFFECTS OF COAL LEACHATE ON LIVER PARAMETERS OF RAINBOW TROUT[a]



[a] Rainbow trout were exposed to coal leachate or to Lake Superior water for 28 days under renewed static conditions. Three fish per exposure were removed at each sampling period and after weight determinations, the livers were pooled and homogenized prior to protein, DNA, and AHH measurements.

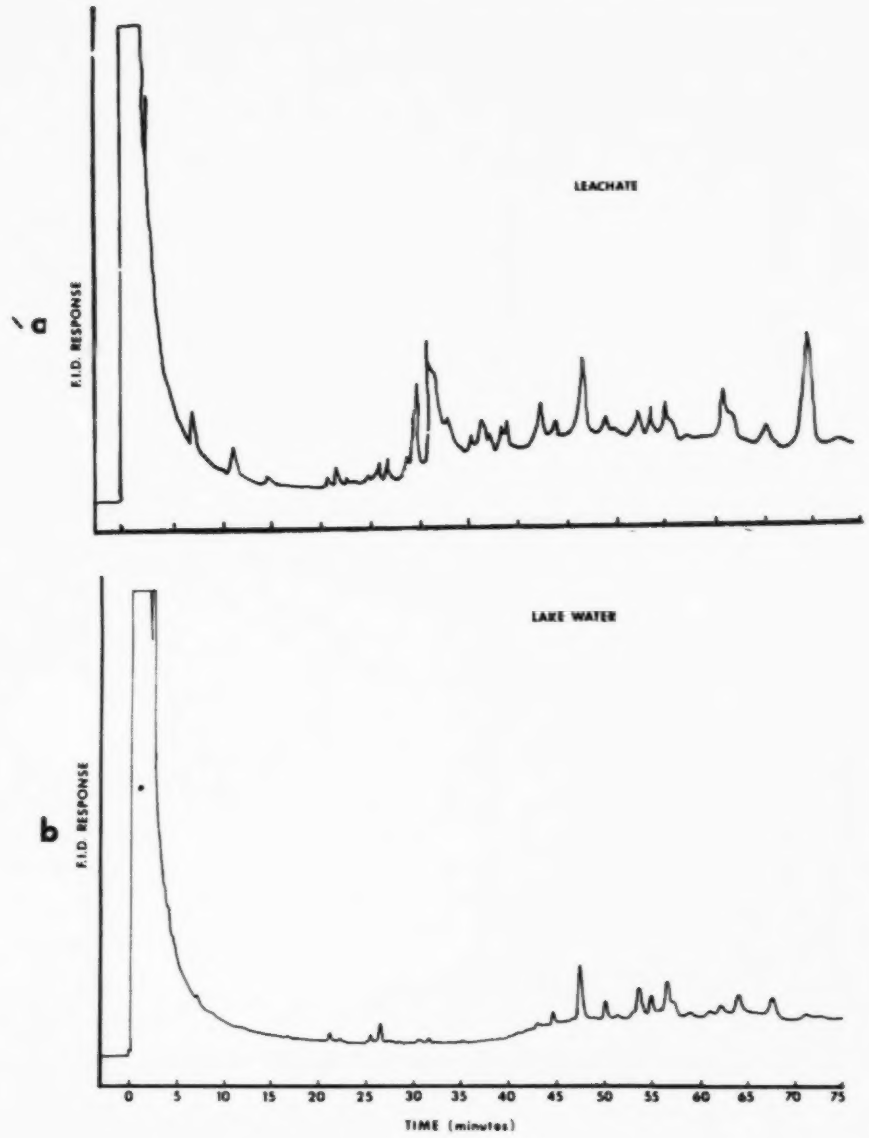
[b] Relative liver weight is expressed as mg liver/g total fish weight.

[c] Protein is the total microsomal protein content/g total liver weight (3 pooled livers).

[d] DNA is the DNA content of the 15,000 g pellet/g total liver weight.

[e] AHH activity is expressed as pM 3-hydroxybenzo(a)pyrene/mg protein/min.

FIGURE A-9
GAS CHROMATOGRAPHIC ANALYSIS OF
FATHEAD MINNOWS EXPOSED TO COAL LEACHATE



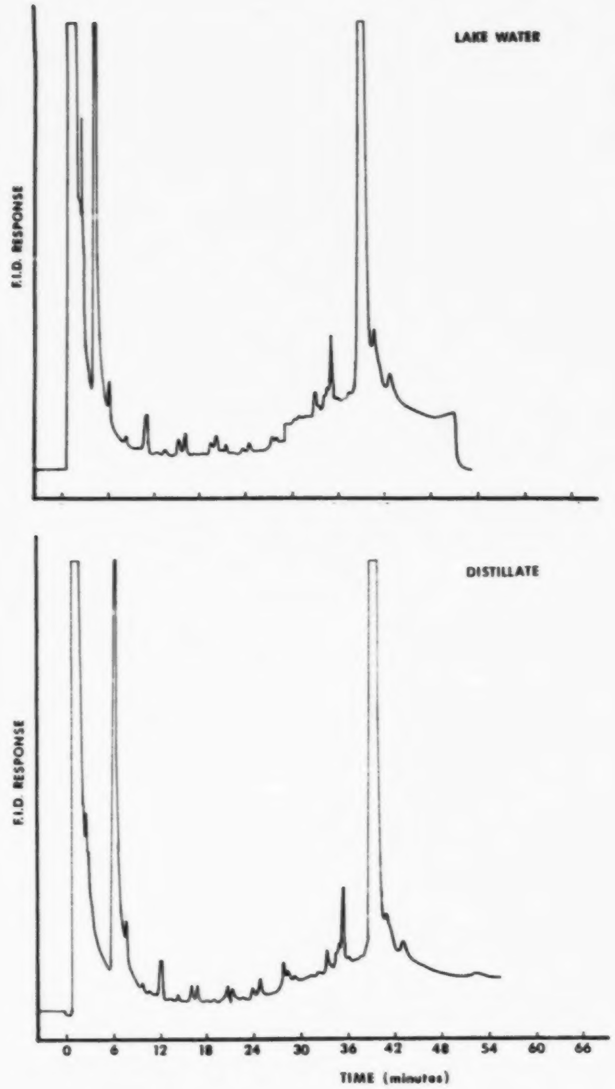
[a] Fathead minnows were exposed to coal leachate (6.3 g coal/L distilled deionized water) for 24 weeks prior to extraction.

[b] Fathead minnows were exposed to XAD-2 purified Lake Superior water for 24 weeks prior to extraction.

GC conditions-column: SP-2250, 2m x 2mm i.d. glass; injector: 250°C; detector 300°C; carrier flow: 20 ml/min; program: 100-235°C at 4°/min.

FIGURE A-10

GAS CHROMATOGRAPHIC ANALYSIS OF RAINBOW
TROUT EXPOSED TO 0.2% COAL DISTILLATE



[a] Rainbow trout were maintained in flowing Lake Superior water for 21 days prior to extraction.

[b] Rainbow trout were exposed to 0.2% coal distillate by metering distillate into flowing Lake Superior water over a 21 day period.

GC conditions-column: 3% OV-101, 2m x 2mm i.d. glass; injector: 250°C; detector: 300°C; carrier flow: 20 ml/min; program: 80-250°C at 4°/min.

APPENDIX B

CALCULATIONS AND ERROR TREATMENT FOR ANALYSIS OF WATER AND FISH TISSUE BY GC/PID

For analysis of each set of unknown solutions a standard curve ($y = a + bx$) was generated by the method of least squares[158] using the data obtained by injecting 1 μℓ of n solutions of known concentration into the GC/PID:

x_i = weight in nanograms of compound in injection of known solution i

y_i = GC peak height or area observed when x_i nanograms were injected.

b = slope of least squares line

a = y-intercept of least squares line

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

$$\bar{y} = \frac{\sum_{i=1}^{n} y_i}{n}$$

$$b = \frac{\left(\sum_{i=1}^{n} x_i y_i\right) - n\bar{x}\bar{y}}{\left(\sum_{i=1}^{n} {x_i}^2\right) - n\bar{x}^2}$$

$$a = \frac{1}{n}\left(\sum_{i=1}^{n} y_i - \sum_{i=1}^{n} x_i\right)$$

The weight of compound x_k in a 1 μℓ injection of unknown solution k which produced a GC peak of area or height y_{kj} on the jth injection is given by:

$$x_k = \left(\frac{\bar{y}_k - a}{b}\right) \pm (s_{xk})$$

where

$$s_{xk} = \sqrt{\frac{s_{y\,x}^{2}}{b^2}\left[\left(\frac{1}{m} + \frac{1}{n}\right) + \frac{(\bar{y}_k - \bar{y})^2}{b^2 \Sigma U^2}\right]}$$

$$s_{y\,x}^{2} = \frac{\Sigma V^2 - b^2 \Sigma U^2}{n-2}$$

$$\Sigma V^2 = \sum_{i=1}^{n} (y_i - \bar{y})^2$$

$$\Sigma U^2 = \sum_{i=1}^{n} (x_i - \bar{x})^2$$

m = number of times unknown solution k was injected

$$\bar{y}_k = \frac{\sum_{i=1}^{m} y_{kj}}{m}$$

The weight of the compound (W) in the total volume of unknown solution k is:

$$W = x_k(M) \pm [s_{xk}(M) + x_k(E)]$$

where

M = volume of unknown solution k in μℓ

E = error in volume determination of unknown solution k. For a solution of volume 1 to 5 ml this error is 0.05 ml.

APPENDIX C

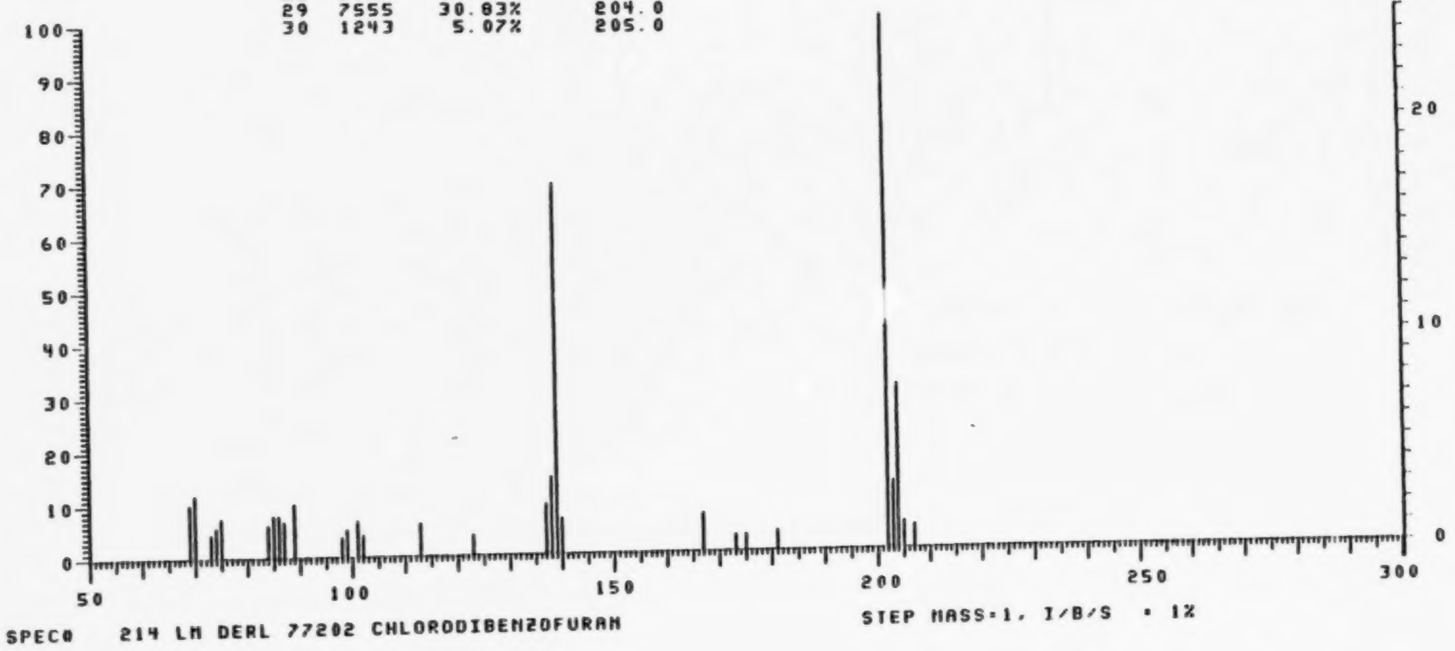
CHLORINATED POLYNUCLEAR AROMATIC HYDROCARBONS: MASS SPECTRAL DATA

```
>>DATE<</SPEC0   214/LM/DERL 77202 CHLORODIBENZOFURAN

 BASE       SUM
24501     94694

PEAK   INT    I/BASE      MASS
   2   2456   10.02%      69.0
   3   2928   11.95%      69.5
   6   1434    5.85%      74.0
   7   1827    7.45%      75.1
   8   1487    6.06%      84.0
   9   1900    7.75%      85.1
  10   1907    7.78%      86.1
  11   1697    6.92%      87.1
  12   2509   10.24%      89.1
  14   1262    5.15%      99.0
  15   1646    6.71%     101.0
  17   1478    6.03%     113.0
  19   2336    9.53%     137.0
  20   3517   14.35%     138.0
  21  17033   69.51%     139.1
  22   1637    6.68%     140.1
  23   1738    7.09%     167.0
  27  24501  100.00%     202.0
  28   3164   12.91%     203.0
  29   7555   30.83%     204.0
  30   1243    5.07%     205.0
```

Electron Energy: 70 ev

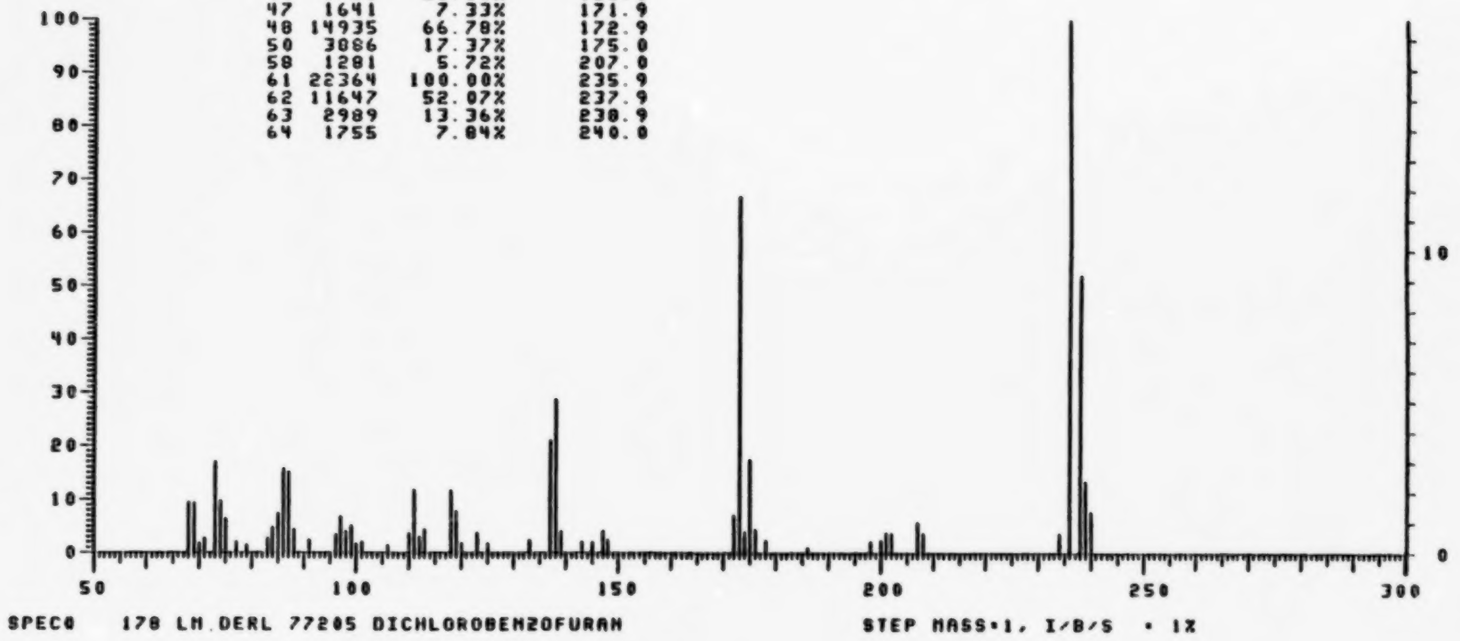
Inlet System: GC

Instrument: Varian CH5



```
>>DATE<</SPEC8   178/LM/DERL 77205 DICHLOROBENZOFURAN

  BASE      SUM
 22364    130392

PEAK    INT    I/BASE      MASS
   1   2158     9.64%      68.0
   2   1131     5.05%      68.6
   3   2162     9.66%      69.2
   7   3852    17.22%      73.4
   8   2183     9.76%      74.4
   9   1449     6.47%      75.4
  14   1674     7.48%      85.0
  15   3546    15.85%      86.1
  16   2023     9.04%      86.5
  17   3382    15.12%      87.1
  23   1493     6.67%      97.0
  25   1149     5.13%      99.0
  30   2680    11.98%     111.0
  33   2641    11.80%     118.0
  34   1755     7.84%     119.0
  39   4723    21.11%     137.0
  40   6456    28.86%     138.0
  47   1641     7.33%     171.9
  48  14935    66.78%     172.9
  50   3086    17.37%     175.0
  58   1281     5.72%     207.0
  61  22364   100.00%     235.9
  62  11647    52.07%     237.9
  63   2989    13.36%     238.9
  64   1755     7.84%     240.0
```

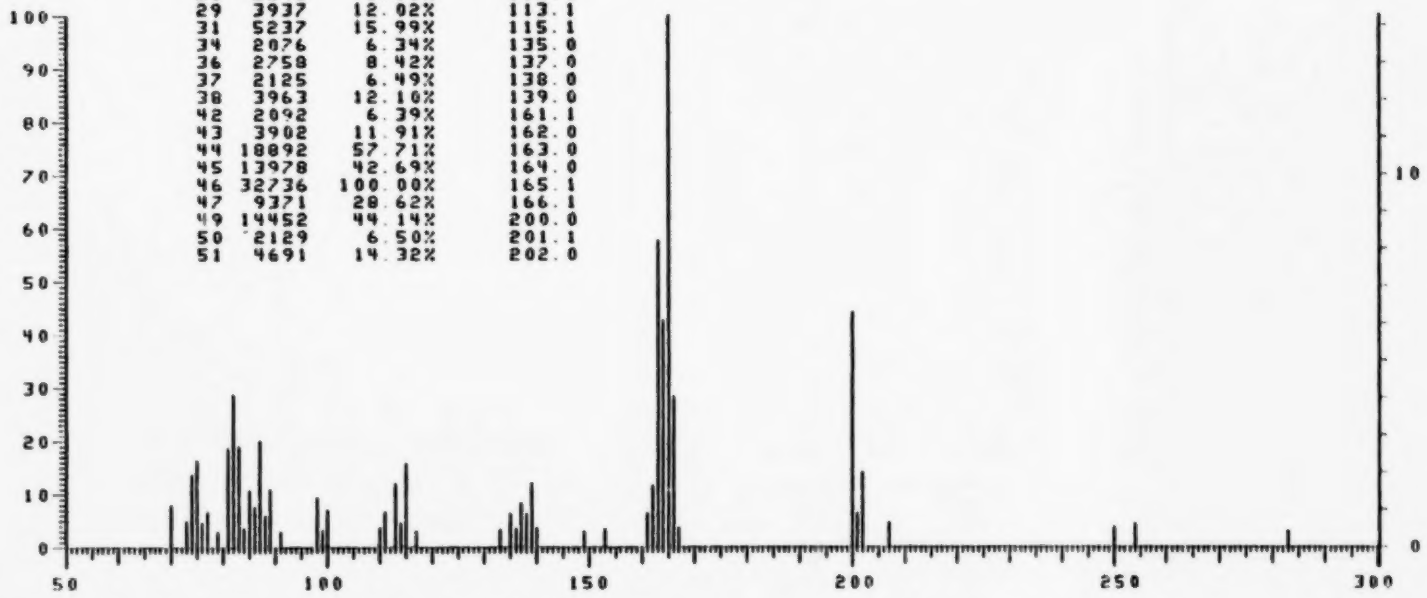
Electron Energy: 70ev
Inlet System: GC
Instrument: Varian CH5



SPEC8   178 LM.DERL 77205 DICHLOROBENZOFURAN   STEP MASS=1, I/B/S = 1X

```
>>DATE<</SPEC0   114/LM/DERL 77176 2-CHLOROFLUORENE

  BASE      SUM
 32736   225996

PEAK    INT    I/BASE      MASS
   1   2706     8.26%      70.5
   2   1700     5.19%      72.6
   3   4497    13.73%      73.8
   4   5446    16.63%      74.8
   6   2264     6.91%      77.0
   9   6144    18.76%      81.1
  10   8998    27.48%      81.6
  11   9414    28.75%      82.1
  12   6258    19.11%      82.6
  13   2776     8.47%      83.0
  16   3519    10.74%      85.0
  17   2599     7.93%      86.1
  19   6566    20.05%      87.1
  20   1991     6.08%      88.1
  22   3646    11.13%      89.1
  24   3095     9.45%      98.0
  26   2368     7.23%     100.1
  28   2248     6.86%     111.0
  29   3937    12.02%     113.1
  31   5237    15.99%     115.1
  34   2076     6.34%     135.0
  36   2758     8.42%     137.0
  37   2125     6.49%     138.0
  38   3963    12.10%     139.0
  42   2092     6.39%     161.1
  43   3902    11.91%     162.0
  44  18892    57.71%     163.0
  45  13978    42.69%     164.0
  46  32736   100.00%     165.1
  47   9371    28.62%     166.1
  49  14452    44.14%     200.0
  50   2129     6.50%     201.1
  51   4691    14.32%     202.0
```

Electron Energy: 70ev
Inlet System: GC
Instrument: Varian CH5



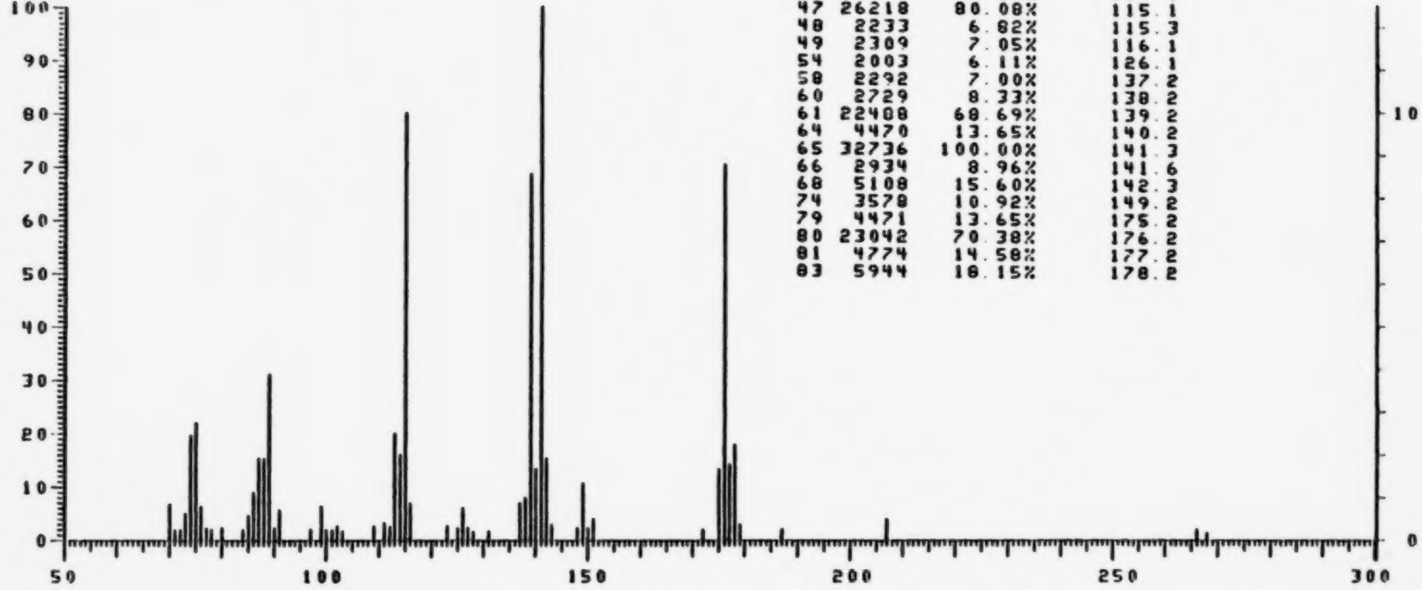
SPEC0   114 LM DERL 77176 2-CHLOROFLUORENE   STEP MASS=1, I/B/S = 1%

Electron Energy: 70ev
Inlet System: GC
Instrument: Varian CH5

CHLOROMETHYLNAPHTHALENE

```
>>DATE<</SPEC0    59/LM/DERL 77182

  BASE       SUM
 32736    259891
```

PEAK	INT	I/BASE	MASS
1	2224	6.79%	70.0
6	1645	5.02%	72.7
9	6472	19.77%	73.8
13	7311	22.33%	74.9
16	2182	6.66%	76.0
22	2987	9.12%	86.1
23	5128	15.66%	87.2
25	5064	15.46%	88.1
27	10181	31.10%	89.2
29	1914	5.84%	91.2
31	2128	6.50%	99.1
43	6650	20.31%	113.1
45	5278	16.12%	114.1
47	26218	80.08%	115.1
48	2233	6.82%	115.3
49	2309	7.05%	116.1
54	2003	6.11%	126.1
58	2292	7.00%	137.2
60	2729	8.33%	138.2
61	22408	68.69%	139.2
64	4470	13.65%	140.2
65	32736	100.00%	141.3
66	2934	8.96%	141.6
68	5108	15.60%	142.3
74	3578	10.92%	149.2
79	4471	13.65%	175.2
80	23042	70.38%	176.2
81	4774	14.58%	177.2
83	5944	18.15%	178.2



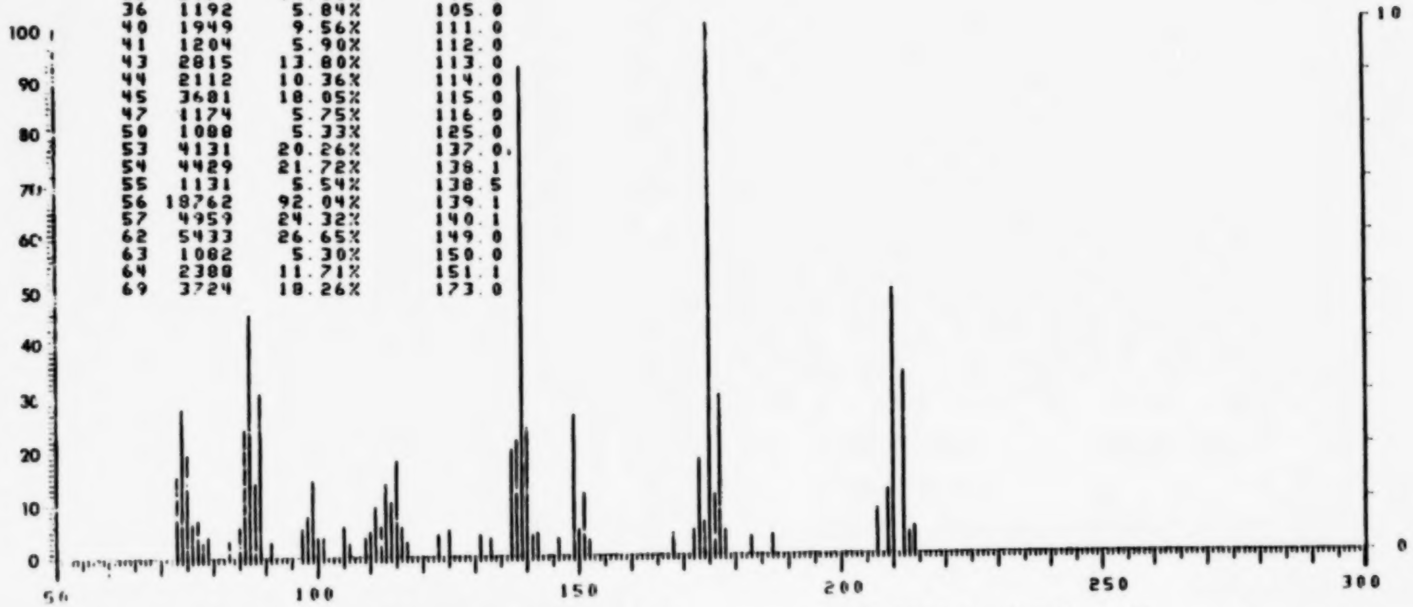
SPEC0   59 LM DERL 77182 CHLOROMETHLYNAPHTHALENE   STEP MASS=1, I/B/S = 1%

```
>>DATE<</SPEC8     87/LM/DERL 77181 DICHLOROMETHYLNAPHTHALENE

  BASE        SUM
 20384     200757

PEAK    INT     I/BASE        MASS         70   1127       5.52%       173.3
   1   3164     15.52%        72.5         71   1323       6.49%       174.0
   3   5724     28.08%        73.7         72  20384     100.00%       175.0
   4   1325      6.50%        74.2         73   2371      11.63%       176.0
   5   3966     19.45%        74.8         75   6124      30.04%       177.0
   6   1211      5.94%        75.2         80   1709       8.38%       207.1
   7   1091      5.35%        75.3         81   2488      12.20%       209.0
   8   1357      6.65%        75.8         82  10134      49.71%       210.0
  11   1468      7.20%        77.0         83   6955      34.11%       212.0
  15   1201      5.89%        85.0         86   1039       5.09%       214.0
  16   1063      5.21%        85.8
  17   4934     24.20%        86.1
  20   1059      5.19%        86.6
  22   9346     45.84%        87.1
  23   1627      7.98%        87.1
  24   1029      5.04%        87.6
  25   2886     14.15%        88.1
  26   1130      5.54%        88.6
  27   6269     30.75%        89.1
  30   1146      5.62%        97.0
  31   1580      7.75%        98.1
  32   2979     14.61%        99.1
  36   1192      5.84%       105.0
  40   1949      9.56%       111.0
  41   1204      5.90%       112.0
  43   2815     13.80%       113.0
  44   2112     10.36%       114.0
  45   3681     18.05%       115.0
  47   1174      5.75%       116.0
  50   1088      5.33%       125.0
  53   4131     20.26%       137.0
  54   4429     21.72%       138.1
  55   1131      5.54%       138.5
  56  18762     92.04%       139.1
  57   4959     24.32%       140.1
  62   5433     26.65%       149.0
  63   1082      5.30%       150.0
  64   2388     11.71%       151.1
  69   3724     18.26%       173.0
```

Electron Energy: 70ev

Inlet System: GC

Instrument: Varian CH5



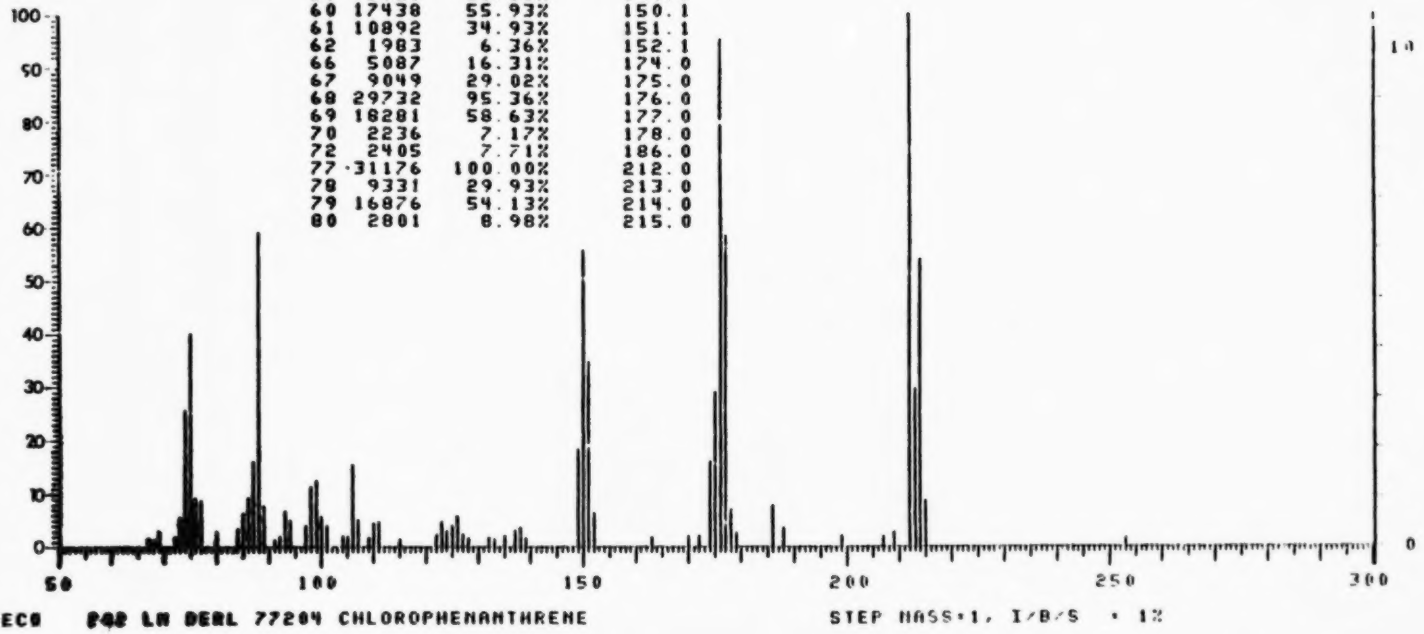
SPEC8   87 LM DERL 77181 DICHLOROMETHYLNAPHTHALENE   STEP MASS=1, I/B/S = 1%

```
>>DATE<</SPEC0   202/LM/DERL 77204 CHLOROPHENANTHRENE

 BASE      SUM
31176    288071

PEAK    INT     I/BASE        MASS
   7   1814      5.81%        73.1
   9   8073     25.89%        74.0
  10  12490     40.06%        75.0
  12   2929      9.39%        76.0
  13   2743      8.79%        77.0
  16   1985      6.36%        85.1
  17   2919      9.36%        86.1
  18   5057     16.22%        87.1
  20   3629     11.64%        87.6
  21  18437     59.13%        88.1
  23   2398      7.69%        88.6
  24   1707      5.47%        89.0
  27   2118      6.79%        93.0
  30   1653      5.30%        94.0
  32   3536     11.34%        98.0
  33   3898     12.50%        99.0
  34   1855      5.95%       100.0
  38   4801     15.39%       106.0
  40   1614      5.17%       107.0
  50   1775      5.69%       126.0
  59   5820     18.66%       149.1
  60  17438     55.93%       150.1
  61  10892     34.93%       151.1
  62   1983      6.36%       152.1
  66   5087     16.31%       174.0
  67   9049     29.02%       175.0
  68  29732     95.36%       176.0
  69  18281     58.63%       177.0
  70   2236      7.17%       178.0
  72   2405      7.71%       186.0
  77 ·31176    100.00%       212.0
  78   9331     29.93%       213.0
  79  16876     54.13%       214.0
  80   2801      8.98%       215.0
```

Electron Energy: 70ev

Inlet System: GC

Instrument: Varian CH5

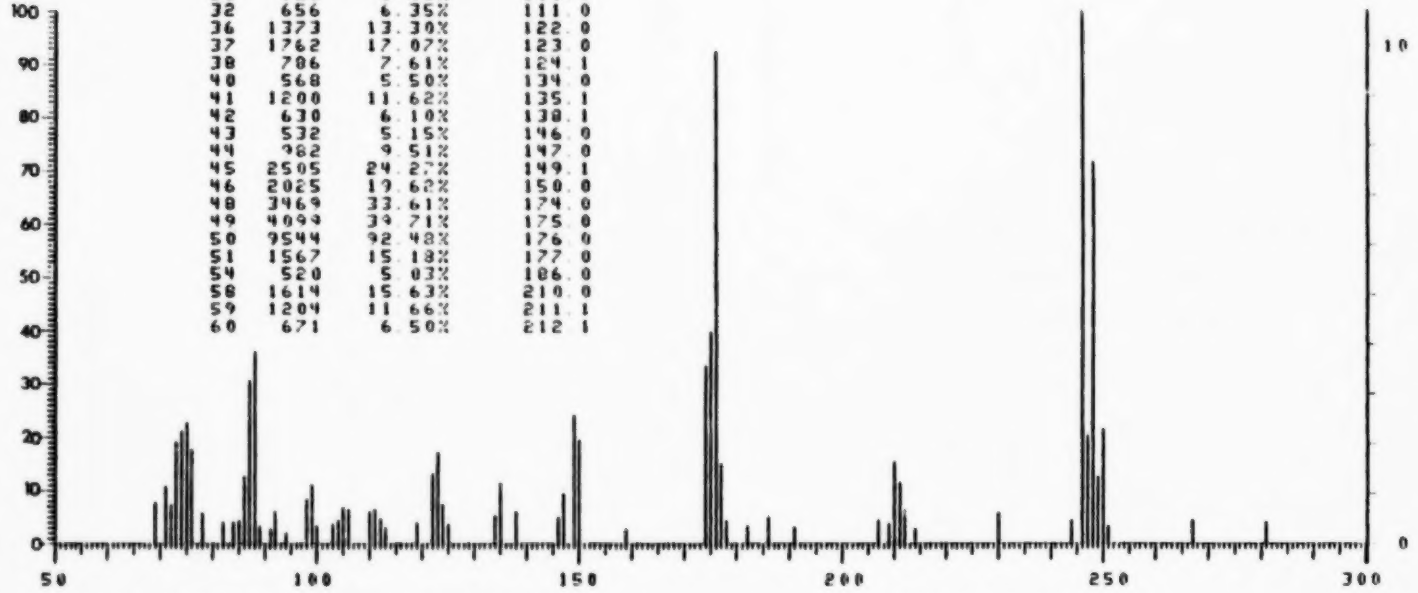


```
>>DATE<</SPEC0   207/LM/DERL 77206 DICHLOROPHENANTHRENE

 BASE       SUM
10320     97586

PEAK    INT    I/BASE      MASS
   1    809     7.83%      69.0     62    616     5.96%     230.1
   2   1134    10.98%      71.0     64  10320   100.00%     246.0
   3    791     7.66%      71.9     65   2118    20.52%     247.0
   4   1993    19.31%      73.0     66   7430    71.99%     247.9
   5   2182    21.14%      74.0     67   1319    12.78%     249.0
   6   2352    22.79%      75.0     68   2218    21.49%     250.0
   7   1840    17.82%      76.0
   8    600     5.81%      78.1
  12   1331    12.89%      86.1
  14   3171    30.72%      87.0
  15   1638    15.87%      87.6
  16    562     5.44%      87.8
  17   3750    36.33%      88.1
  20    641     6.21%      92.0
  22    886     8.58%      98.0
  24   1151    11.15%      99.0
  28    695     6.73%     105.0
  29    658     6.37%     105.5
  30    675     6.54%     106.0
  31    623     6.03%     110.0
  32    656     6.35%     111.0
  36   1373    13.30%     122.0
  37   1762    17.07%     123.0
  38    786     7.61%     124.1
  40    568     5.50%     134.0
  41   1200    11.62%     135.1
  42    630     6.10%     138.1
  43    532     5.15%     146.0
  44    982     9.51%     147.0
  45   2505    24.27%     149.1
  46   2025    19.62%     150.0
  48   3469    33.61%     174.0
  49   4099    39.71%     175.0
  50   9544    92.48%     176.0
  51   1567    15.18%     177.0
  54    520     5.03%     186.0
  58   1614    15.63%     210.0
  59   1204    11.66%     211.1
  60    671     6.50%     212.1
```

Electron Energy: 70ev

Inlet System: GC

Instrument: Varian CH5



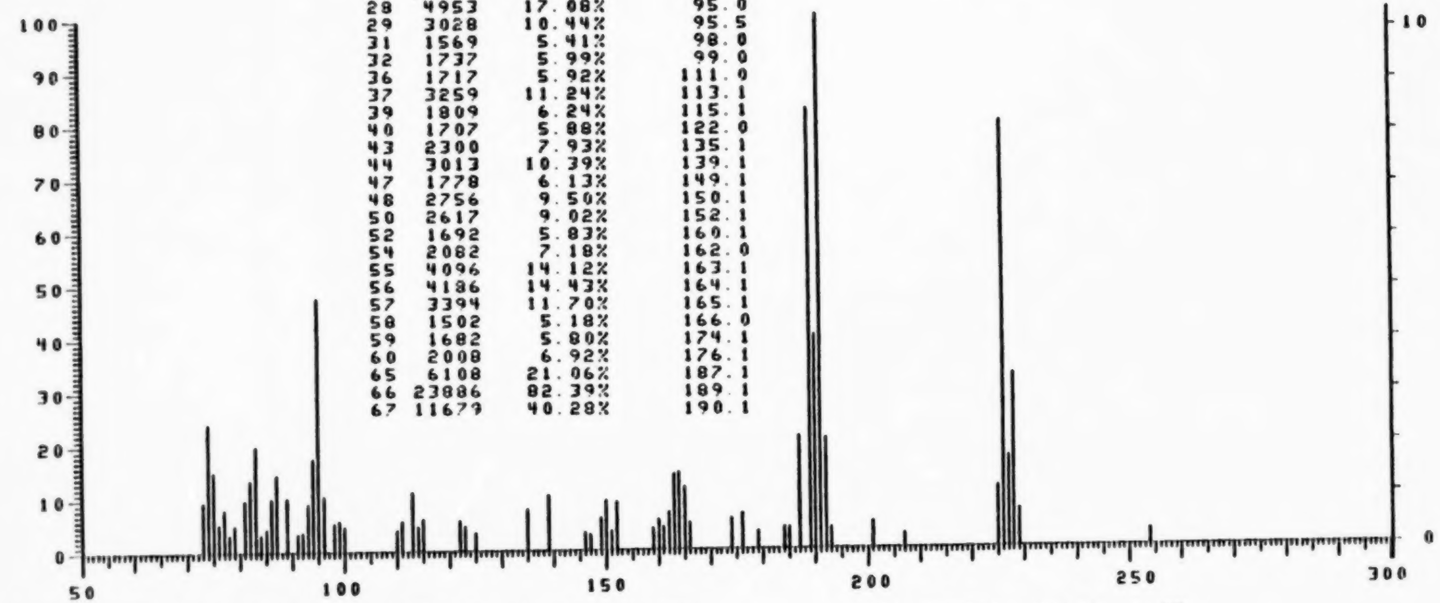
SPEC0   207 LM DERL 77206 DICHLOROPHENANTHRENE   STEP MASS=1, I/B/S = 1%

```
>>DATE<</SPEC#   183/LM/DERL 77179 CHLOROMETHYLPHENANTHRENE

 BASE      SUM
28990    280197

PEAK    INT    I/BASE         MASS         68 28990   100.00%       191.1
   1   2776     9.57%         72.8         69  6029    20.79%       192.1
   2   7036    24.27%         73.8         71  1481     5.10%       201.0
   3   4382    15.11%         74.8         73  3321    11.45%       225.1
   4   1606     5.53%         75.9         74 23120    79.75%       226.1
   5   2340     8.07%         77.0         75  4993    17.22%       227.1
   7   1488     5.13%         79.0         76  9378    32.34%       228.1
   8   2847     9.82%         81.1         77  2065     7.12%       229.0
   9   3917    13.51%         81.6
  10   3615    12.46%         82.1
  11   5742    19.80%         82.6
  15   2992    10.32%         86.1
  17   4182    14.42%         87.1
  18   2967    10.23%         89.1
  21   1489     5.13%         92.5
  22   2679     9.24%         93.0
  23   4961    17.11%         93.6
  24   1537     5.30%         93.6
  25   5029    17.34%         94.0
  26   1507     5.19%         94.1
  27  13792    47.57%         94.6
  28   4953    17.08%         95.0
  29   3028    10.44%         95.5
  31   1569     5.41%         98.0
  32   1737     5.99%         99.0
  36   1717     5.92%        111.0
  37   3259    11.24%        113.1
  39   1809     6.24%        115.1
  40   1707     5.88%        122.0
  43   2300     7.93%        135.1
  44   3013    10.39%        139.1
  47   1778     6.13%        149.1
  48   2756     9.50%        150.1
  50   2617     9.02%        152.1
  52   1692     5.83%        160.1
  54   2082     7.18%        162.0
  55   4096    14.12%        163.1
  56   4186    14.43%        164.1
  57   3394    11.70%        165.1
  58   1502     5.18%        166.0
  59   1682     5.80%        174.1
  60   2008     6.92%        176.1
  65   6108    21.06%        187.1
  66  23886    82.39%        189.1
  67  11679    40.28%        190.1
```

Electron Energy: 70ev

Inlet System: GC

Instrument: Varian CH5



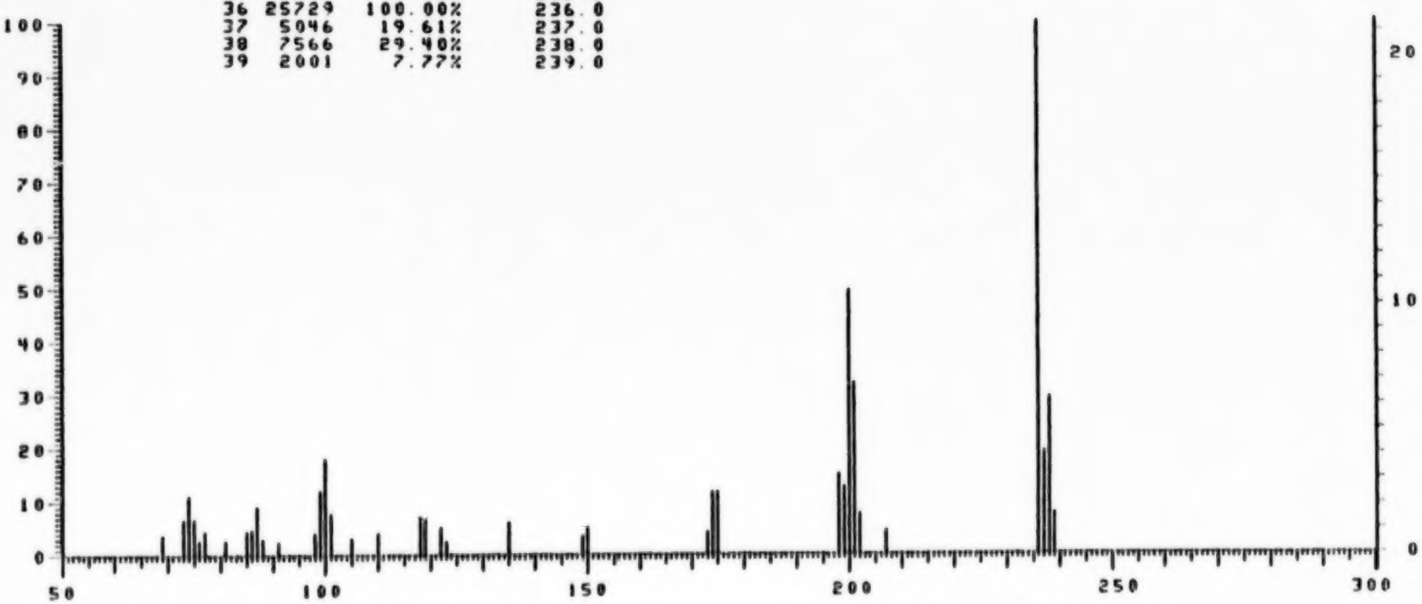
SPEC#   183 LM DERL 77179 CHLOROMETHYLPHENANTHRENE   STEP MASS=1, I/B/S = 1%

```
>>DATE<</SPEC0   273/LM/DERL 77203 CHLOROFLUORANTHENE

  BASE      SUM
 25729   118899

PEAK    INT     I/BASE       MASS
   2   1786      6.94%       73.0
   3   2863     11.12%       73.9
   4   1745      6.78%       75.0
  10   2386      9.27%       87.1
  14   3170     12.32%       99.0
  15   1536      5.96%       99.5
  16   4698     18.25%      100.0
  17   2000      7.77%      100.5
  20   1865      7.24%      118.0
  21   1726      6.70%      119.0
  22   1362      5.29%      122.0
  24   1585      6.16%      135.0
  26   1303      5.06%      150.1
  28   3081     11.97%      174.0
  29   3068     11.92%      175.0
  30   3867     15.02%      198.0
  31   3281     12.75%      199.0
  32  12772     49.64%      200.0
  33   8277     32.16%      201.1
  34   2005      7.79%      202.1
  36  25729    100.00%      236.0
  37   5046     19.61%      237.0
  38   7566     29.40%      238.0
  39   2001      7.77%      239.0
```

Electron Energy: 70ev

Inlet System: GC

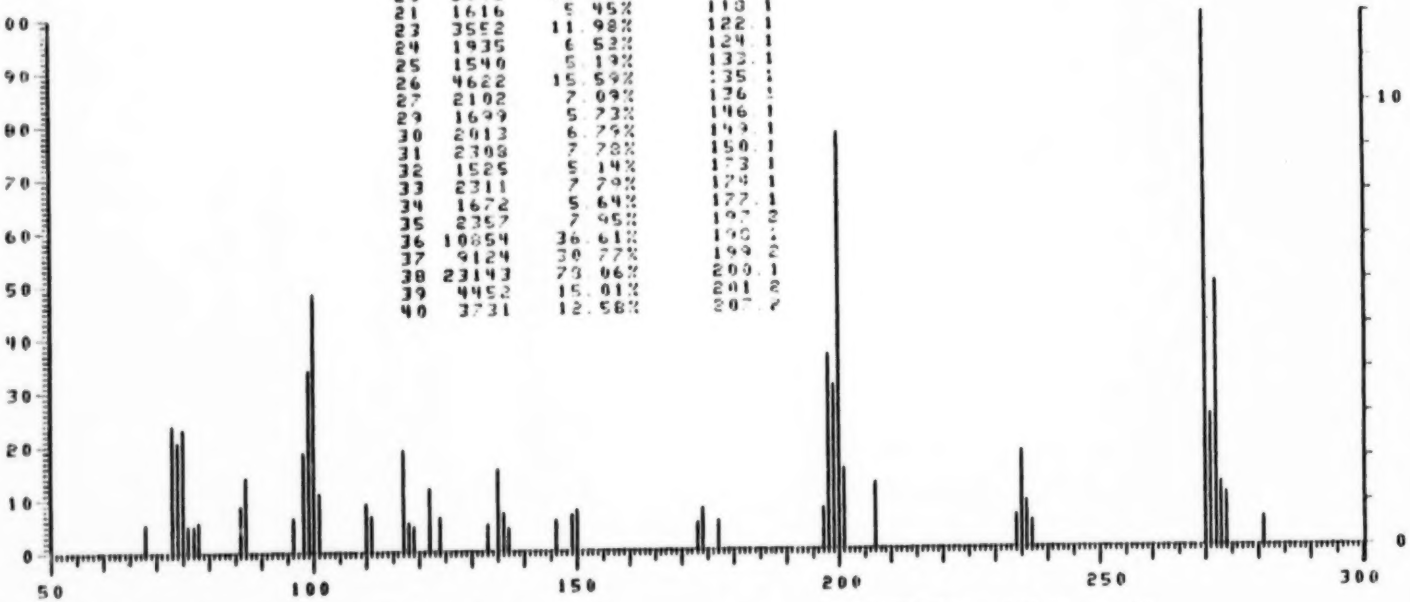
Instrument: Varian CH5



SPEC0   273 LM DERL 77203 CHLOROFLUORANTHENE   STEP MASS=1. I/B/S = 1%

Reproduced from best available copy.

```
>>DATE<</SPECA   241/LM/DERL 77207

  BASE       SUM          DICHLOROFLUORANTHENE
 29644    244425

PEAK    INT     I/BASE       MASS
   1    1588     5.35%       68.4     41    1824     6.15%     234.3
   2    7103    23.96%       72.8     42    5435    18.33%     235.1
   3    6198    20.90%       73.8     43    2613     8.81%     236.2
   4    6915    23.28%       74.9     44    1539     5.19%     237.2
   5    1538     5.18%       76.0     45   29644   100.00%     270.2
   6    1531     5.16%       77.0     46    7387    24.91%     271.3
   7    1681     5.67%       78.1     47   14796    49.91%     272.2
   8    2641     8.90%       85.1     48    3604    12.15%     273.3
   9    1594     5.37%       86.6     49    3014    10.16%     274.2
  10    4244    14.31%       87.1     50    1597     5.38%     281.3
  11    1883     6.35%       96.1
  12    5554    18.73%       98.1
  13   10130    34.17%       99.1     Electron Energy:     70ev
  14    1601     5.40%   [illegible]
  15    7422    25.03%       99.6     Inlet System:        GC
  16   14416    48.63%      100.1
  17    3263    11.00%      100.6     Instrument:          Varian CH5
  18    2670     9.00%      110.1
  19    1992     6.71%      111.1
  20    5646    19.04%      117.1
  21    1616     5.45%      118.1
  23    3552    11.98%      122.1
  24    1935     6.52%      124.1
  25    1540     5.19%      133.1
  26    4622    15.59%      135.1
  27    2102     7.09%      136.1
  29    1699     5.73%      146.1
  30    2013     6.79%      149.1
  31    2308     7.78%      150.1
  32    1525     5.14%      173.1
  33    2311     7.79%      174.1
  34    1672     5.64%      177.1
  35    2357     7.95%      197.2
  36   10854    36.61%      198.2
  37    9124    30.77%      199.2
  38   23143    78.06%      200.1
  39    4452    15.01%      201.2
  40    3731    12.58%      207.2
```



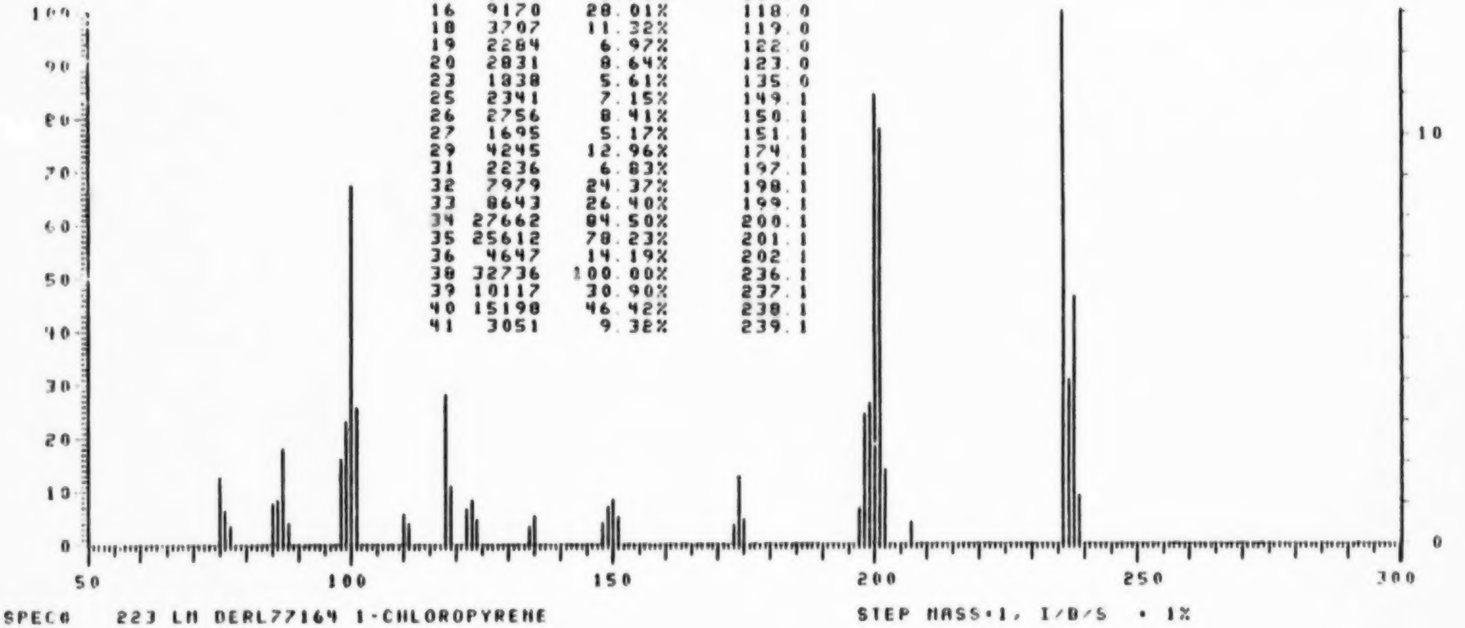
SPECA   241 LM DERL 77207 DICHLOROFLUORANTHENE   STEP MASS=1, I/B/S = 1%

```
>>DATE<</SPEC8   223/LM/DERL77164 1-CHLOROPYRENE

  BASE      SUM
 32736    253210

PEAK    INT    I/BASE       MASS
   1   4198    12.82%       74.0
   2   2172     6.63%       75.9
   4   2581     7.88%       85.1
   5   2809     8.58%       86.1
   6   6000    18.32%       87.1
   8   5319    16.24%       98.0
   9   7610    23.24%       99.0
  10   4310    13.16%       99.6
  11  22110    67.54%      100.1
  12   8501    25.96%      100.6
  13   3613    11.03%      101.1
  14   1870     5.71%      110.0
  16   9170    28.01%      118.0
  18   3707    11.32%      119.0
  19   2284     6.97%      122.0
  20   2831     8.64%      123.0
  23   1838     5.61%      135.0
  25   2341     7.15%      149.1
  26   2756     8.41%      150.1
  27   1695     5.17%      151.1
  29   4245    12.96%      174.1
  31   2236     6.83%      197.1
  32   7979    24.37%      198.1
  33   8643    26.40%      199.1
  34  27662    84.50%      200.1
  35  25612    78.23%      201.1
  36   4647    14.19%      202.1
  38  32736   100.00%      236.1
  39  10117    30.90%      237.1
  40  15198    46.42%      238.1
  41   3051     9.32%      239.1
```

Electron Energy: 70ev

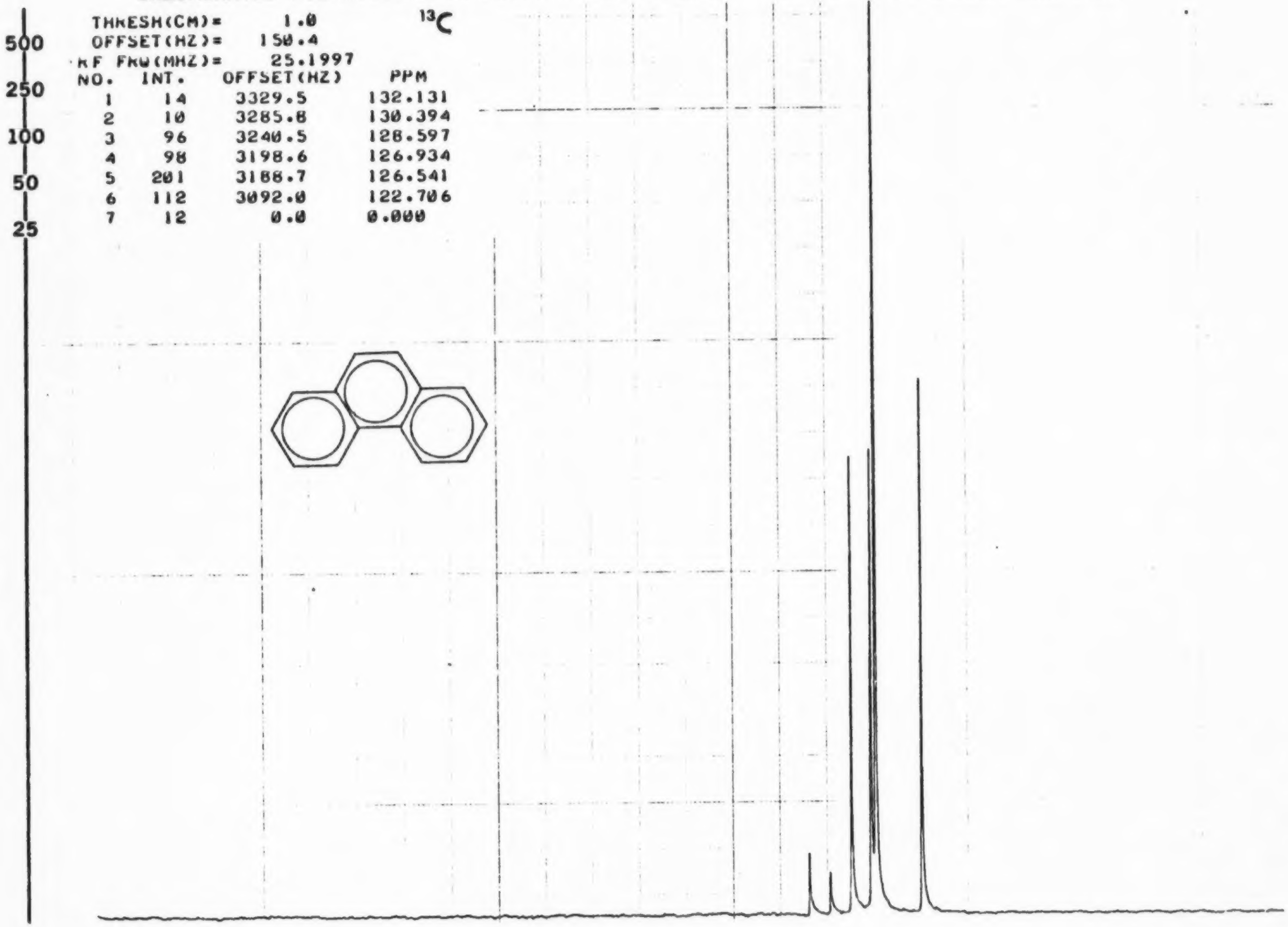
Inlet System: GC

Instrument: Varian CH5

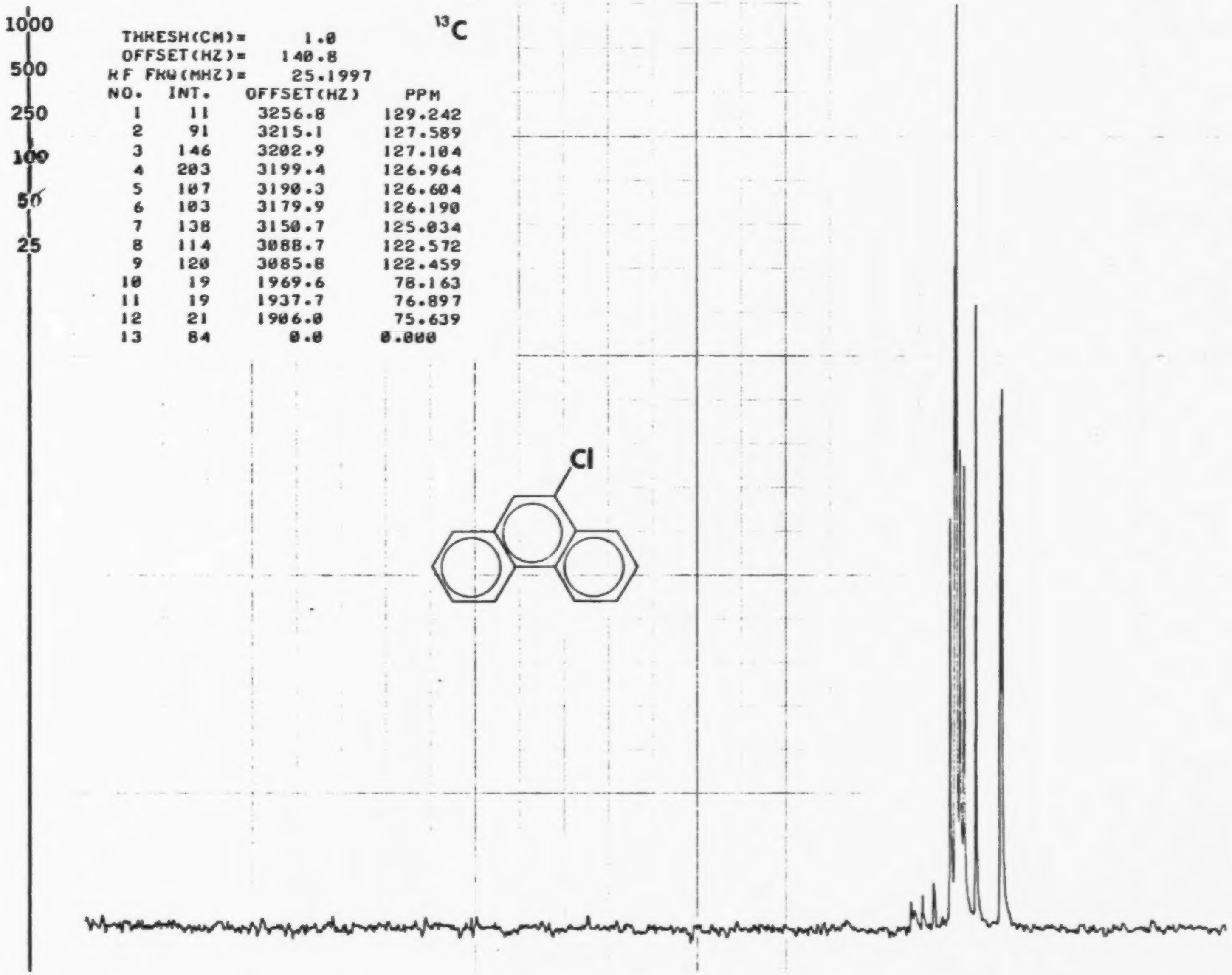


APPENDIX D

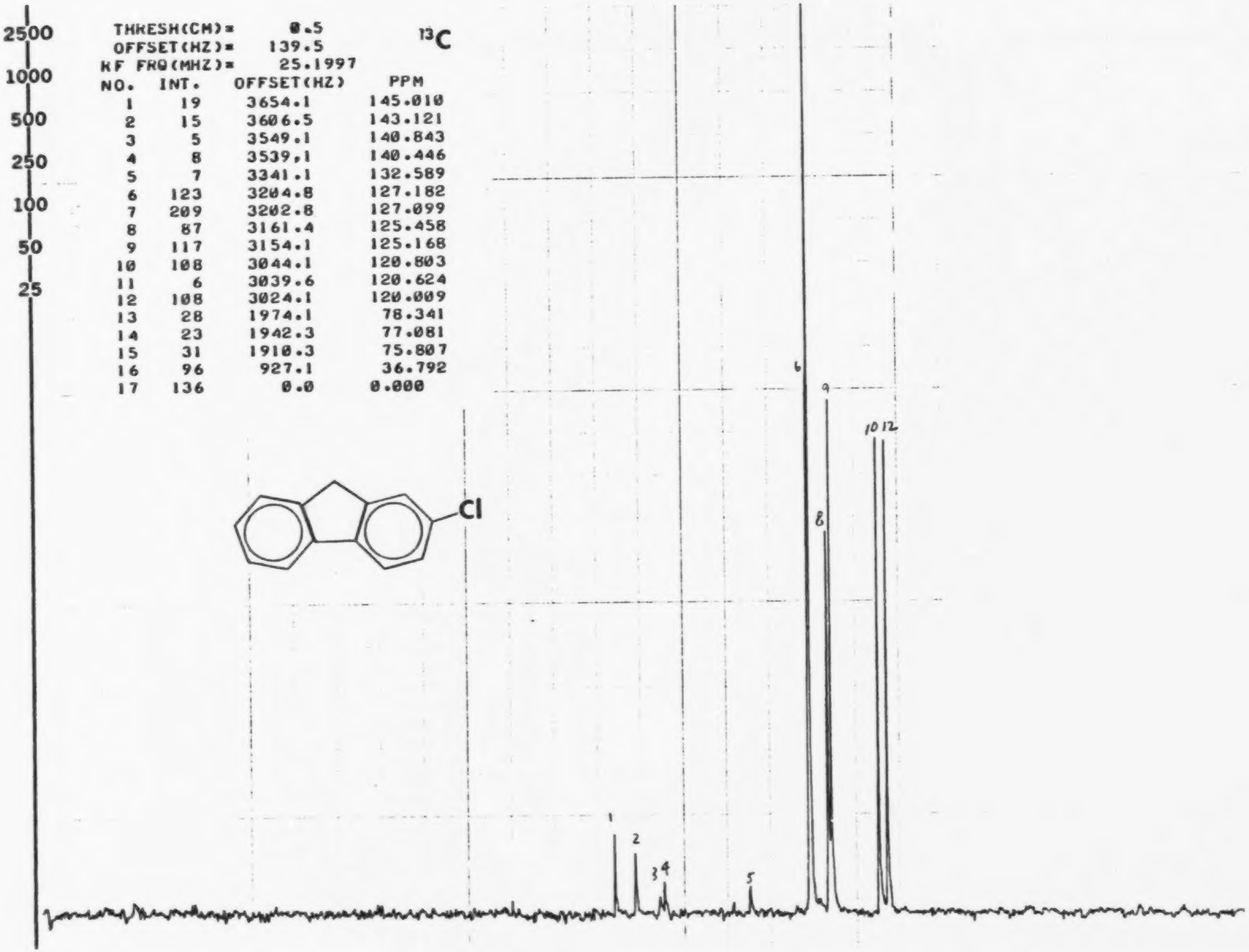
CHLORINATED POLYNUCLEAR AROMATIC HYDROCARBONS: ^{13}C and ^{1}H (100 MHz) NMR DATA



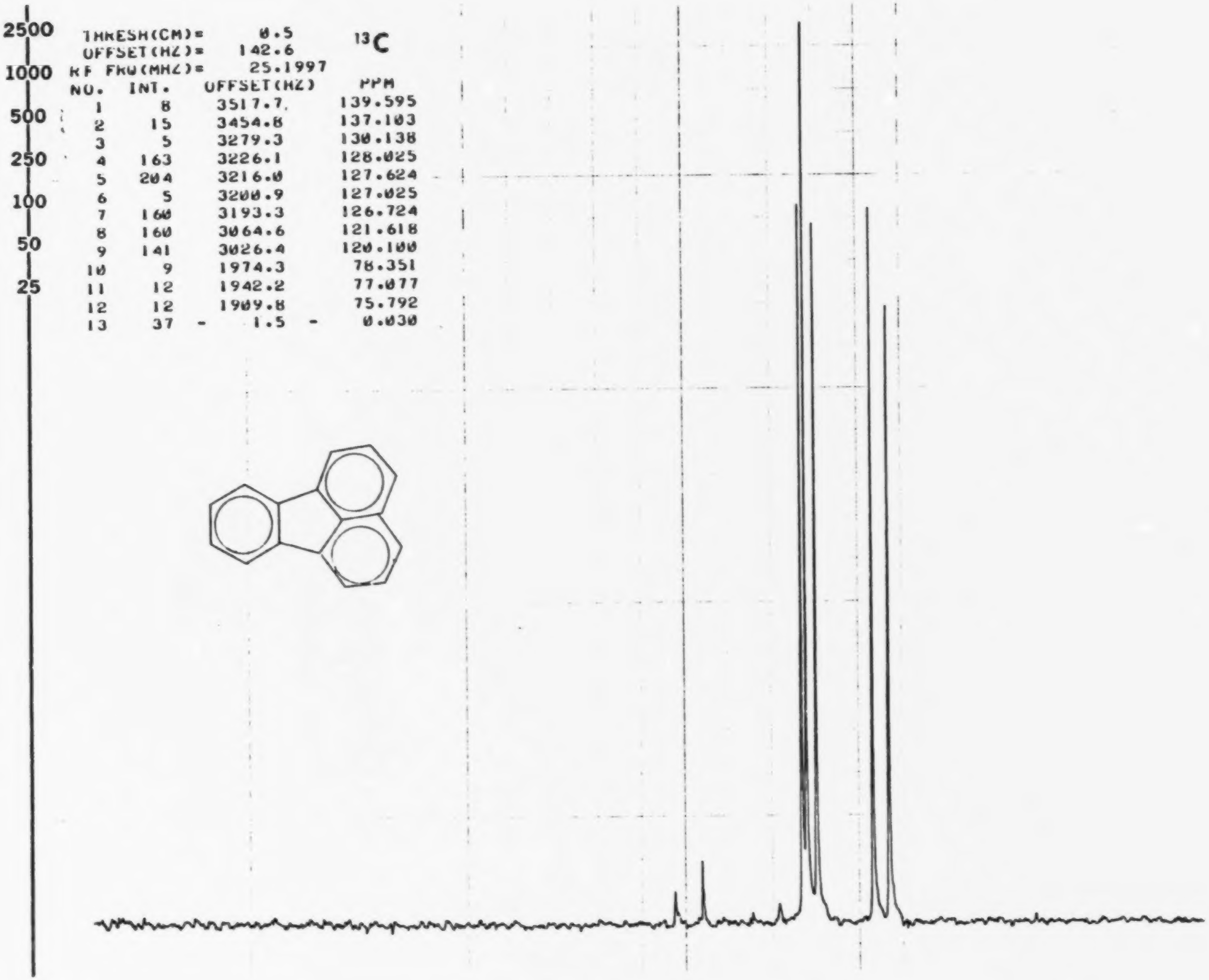
```
THRESH(CM)=      1.0
OFFSET(HZ)=    150.4
RF FRQ(MHZ)=      25.1997
NO.  INT.    OFFSET(HZ)     PPM
  1    14      3329.5     132.131
  2    10      3285.8     130.394
  3    96      3240.5     128.597
  4    98      3198.6     126.934
  5   201      3188.7     126.541
  6   112      3092.0     122.706
  7    12         0.0       0.000
```



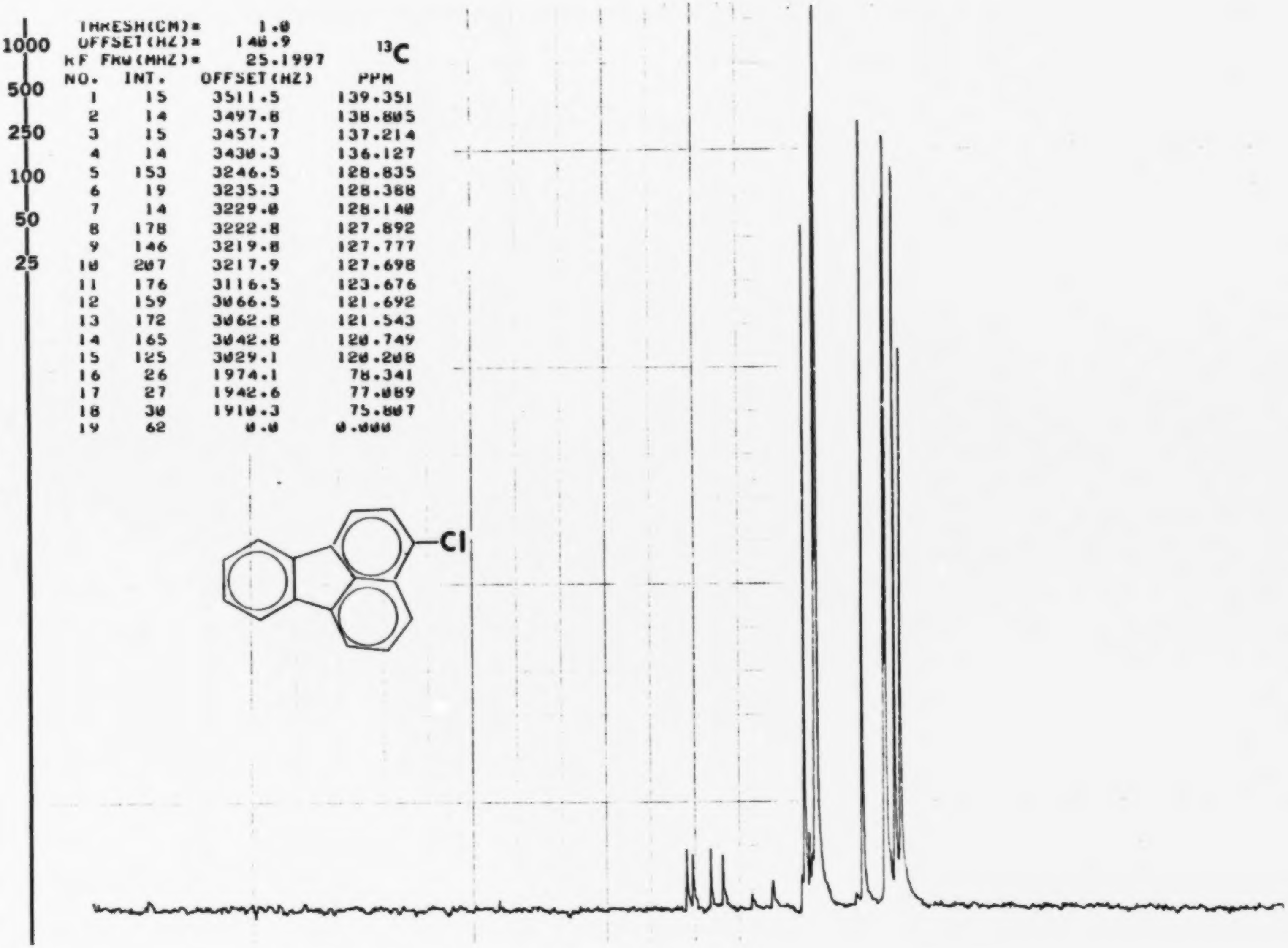
```
THRESH(CM)=      1.0
OFFSET(HZ)=    140.8
RF FRQ(MHZ)=      25.1997
NO.  INT.    OFFSET(HZ)      PPM
  1    11      3256.8      129.242
  2    91      3215.1      127.589
  3   146      3202.9      127.104
  4   203      3199.4      126.964
  5   107      3190.3      126.604
  6   103      3179.9      126.190
  7   138      3150.7      125.034
  8   114      3088.7      122.572
  9   120      3085.8      122.459
 10    19      1969.6       78.163
 11    19      1937.7       76.897
 12    21      1906.0       75.639
 13    84         0.0       0.000
```



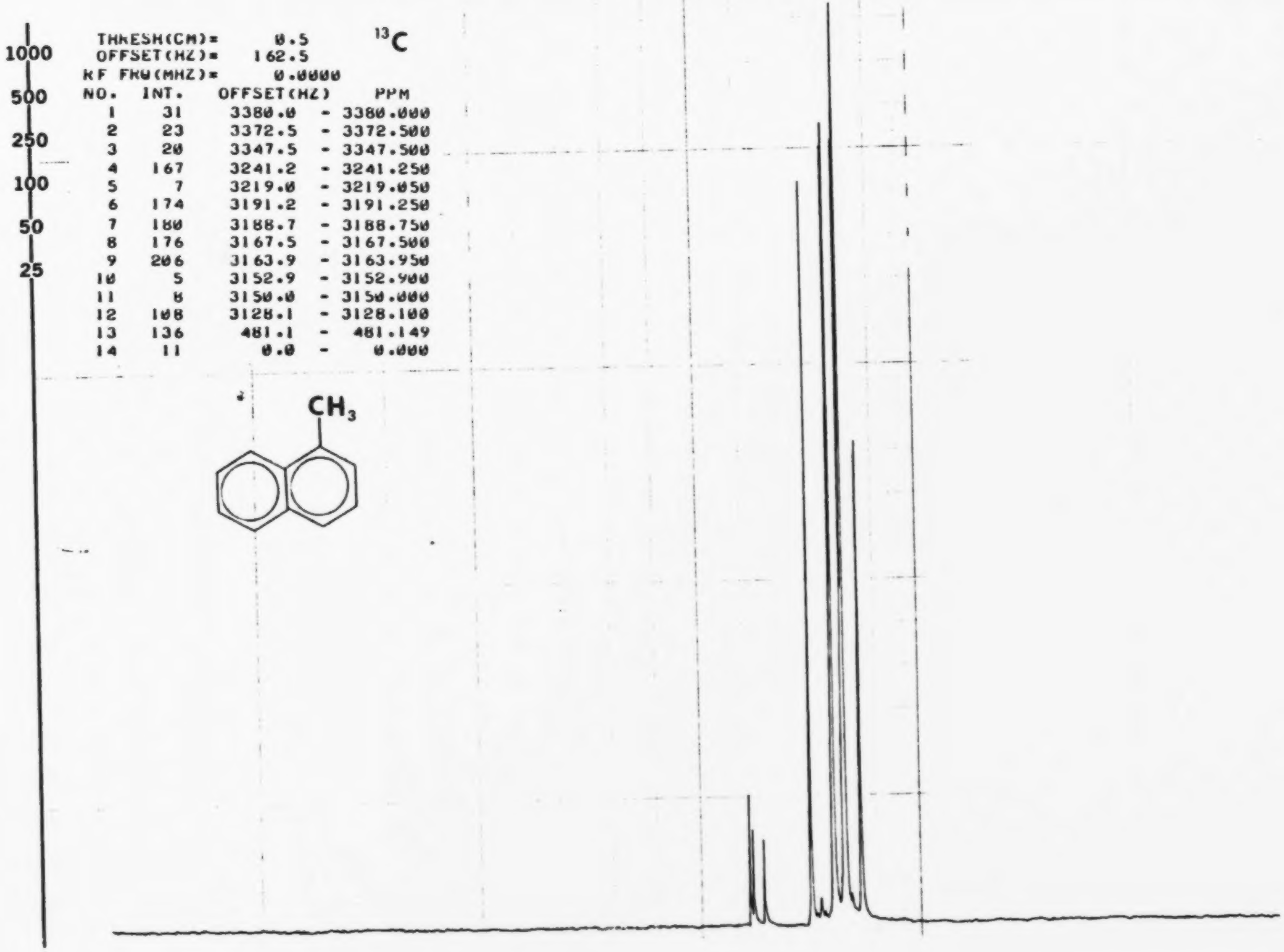
```
 THRESH(CM)=      0.5
 OFFSET(HZ)=    139.5
RF FRQ(MHZ)=      25.1997
NO.  INT.   OFFSET(HZ)     PPM
  1    19     3654.1     145.010
  2    15     3606.5     143.121
  3     5     3549.1     140.843
  4     8     3539,1     140.446
  5     7     3341.1     132.589
  6   123     3204.8     127.182
  7   209     3202.8     127.099
  8    87     3161.4     125.458
  9   117     3154.1     125.168
 10   108     3044.1     120.803
 11     6     3039.6     120.624
 12   108     3024.1     120.009
 13    28     1974.1      78.341
 14    23     1942.3      77.081
 15    31     1910.3      75.807
 16    96      927.1      36.792
 17   136        0.0     0.000
```



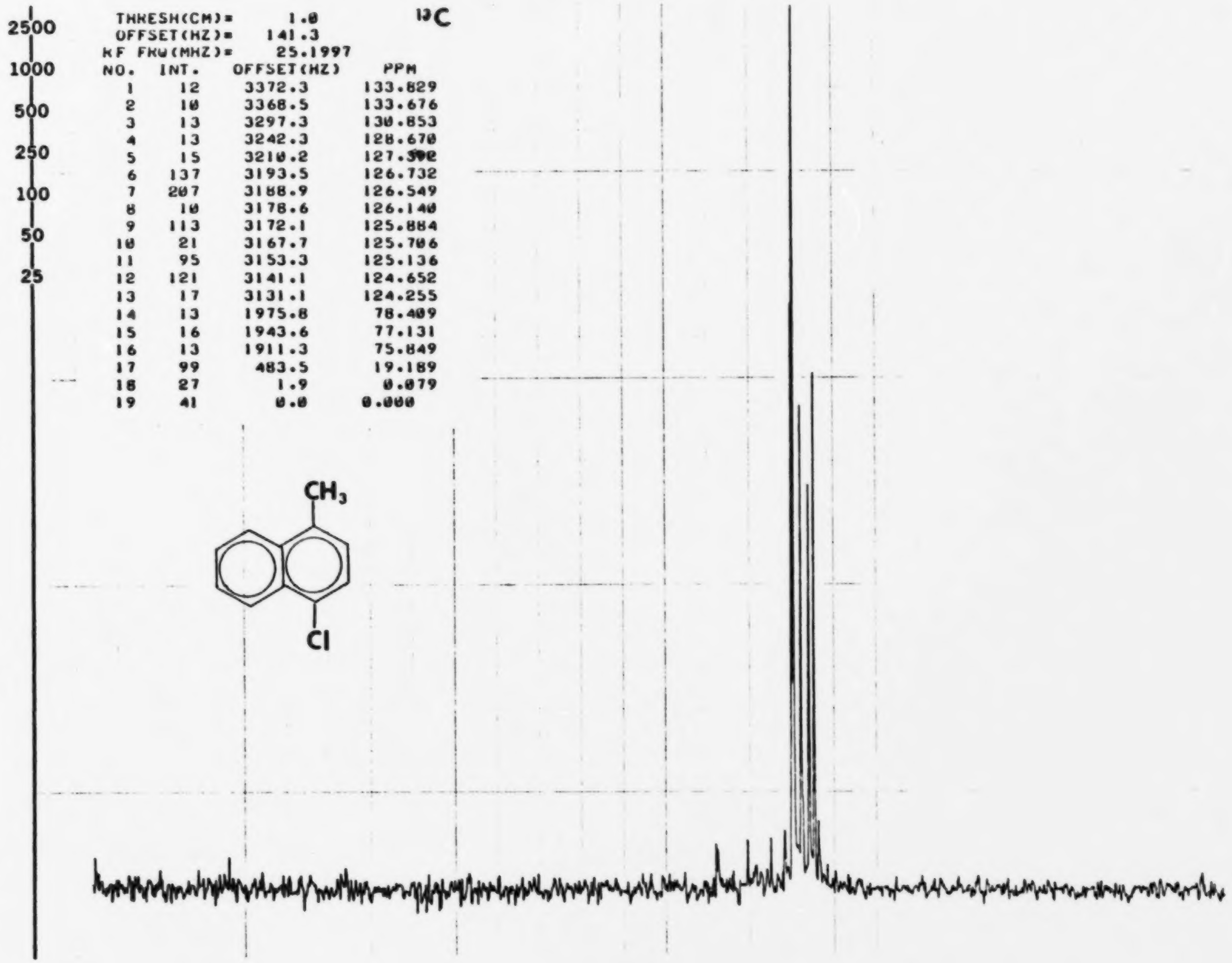
```
THRESH(CM)=      0.5
OFFSET(HZ)=    142.6
RF FRQ(MHZ)=      25.1997
NO.  INT.   OFFSET(HZ)     PPM
  1     8    3517.7.    139.595
  2    15    3454.8     137.103
  3     5    3279.3     130.138
  4   163    3226.1     128.025
  5   204    3216.0     127.624
  6     5    3200.9     127.025
  7   160    3193.3     126.724
  8   160    3064.6     121.618
  9   141    3026.4     120.100
 10     9    1974.3      78.351
 11    12    1942.2      77.077
 12    12    1909.8      75.792
 13    37  -    1.5  -    0.030
```



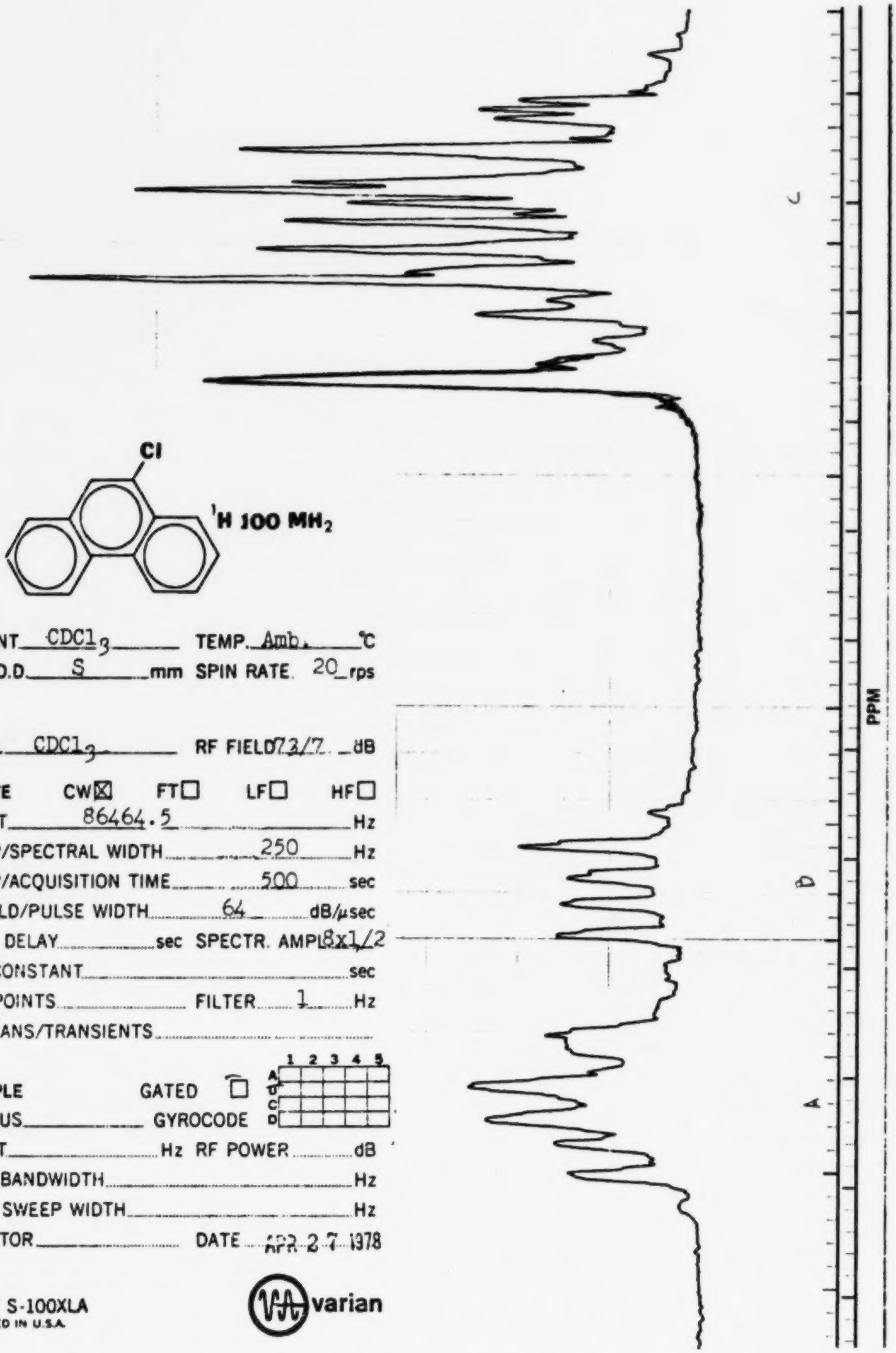
```
THRESH(CM)=      1.0
OFFSET(HZ)=    140.9
RF FRQ(MHZ)=      25.1997
NO.   INT.   OFFSET(HZ)      PPM
  1     15     3511.5      139.351
  2     14     3497.8      138.805
  3     15     3457.7      137.214
  4     14     3430.3      136.127
  5    153     3246.5      128.835
  6     19     3235.3      128.388
  7     14     3229.0      128.140
  8    178     3222.8      127.892
  9    146     3219.8      127.777
 10    207     3217.9      127.698
 11    176     3116.5      123.676
 12    159     3066.5      121.692
 13    172     3062.8      121.543
 14    165     3042.8      120.749
 15    125     3029.1      120.208
 16     26     1974.1       78.341
 17     27     1942.6       77.089
 18     30     1910.3       75.807
 19     62        0.0      0.000
```



```
 THRESH(CM)=       0.5
 OFFSET(HZ)=     162.5
RF FRQ(MHZ)=        0.0000
NO.   INT.    OFFSET(HZ)       PPM
  1     31     3380.0   -  3380.000
  2     23     3372.5   -  3372.500
  3     20     3347.5   -  3347.500
  4    167     3241.2   -  3241.250
  5      7     3219.0   -  3219.050
  6    174     3191.2   -  3191.250
  7    180     3188.7   -  3188.750
  8    176     3167.5   -  3167.500
  9    206     3163.9   -  3163.950
 10      5     3152.9   -  3152.900
 11      8     3150.0   -  3150.000
 12    108     3128.1   -  3128.100
 13    136      481.1   -   481.149
 14     11        0.0   -     0.000
```



```
 THRESH(CM)=      1.0          13C
 OFFSET(HZ)=    141.3
RF FRQ(MHZ)=      25.1997
NO.   INT.   OFFSET(HZ)     PPM
  1     12     3372.3     133.829
  2     10     3368.5     133.676
  3     13     3297.3     130.853
  4     13     3242.3     128.670
  5     15     3210.2     127.390
  6    137     3193.5     126.732
  7    207     3188.9     126.549
  8     10     3178.6     126.140
  9    113     3172.1     125.884
 10     21     3167.7     125.706
 11     95     3153.3     125.136
 12    121     3141.1     124.652
 13     17     3131.1     124.255
 14     13     1975.8      78.409
 15     16     1943.6      77.131
 16     13     1911.3      75.849
 17     99      483.5      19.189
 18     27        1.9       0.079
 19     41        0.0     0.000
```



SOLVENT CDCl$_3$ TEMP. Amb. °C

TUBE O.D. 5 mm SPIN RATE 20 rps

LOCK

SIGNAL CDCl$_3$ RF FIELD 73/7 dB

OBSERVE CW☒ FT☐ LF☐ HF☐

OFFSET 86464.5 Hz

SWEEP/SPECTRAL WIDTH 250 Hz

SWEEP/ACQUISITION TIME 500 sec

RF FIELD/PULSE WIDTH 64 dB/μsec

PULSE DELAY sec SPECTR. AMPL 8x1/2

TIME CONSTANT sec

DATA POINTS FILTER 1 Hz

NO. SCANS/TRANSIENTS

DECOUPLE GATED ☐

	1	2	3	4	5
A					
B					
C					
D					

NUCLEUS GYROCODE

OFFSET Hz RF POWER dB

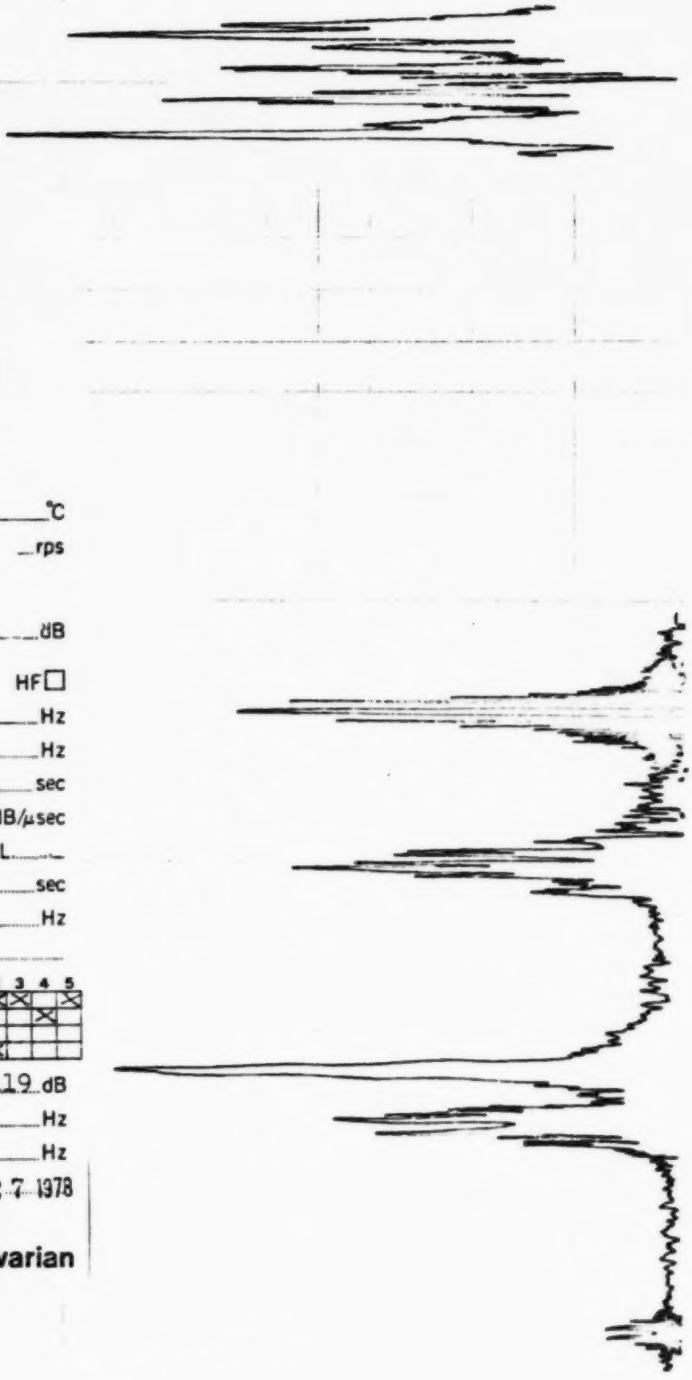
NOISE BANDWIDTH Hz

INDOR SWEEP WIDTH Hz

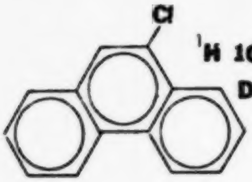
OPERATOR DATE APR 27 1978

CHART S-100XLA
PRINTED IN U.S.A.

varian



Decpl C



^{1}H 100 MH$_2$
Decoupled

SOLVENT______ TEMP______°C
TUBE O.D______mm SPIN RATE ___rps

LOCK
SIGNAL______ RF FIELD______dB

OBSERVE CW☒ FT☐ LF☐ HF☐
OFFSET 86464·5 Hz
SWEEP/SPECTRAL WIDTH 350 Hz
SWEEP/ACQUISITION TIME 500 sec
RF FIELD/PULSE WIDTH______dB/μsec
PULSE DELAY______sec SPECTR. AMPL______
TIME CONSTANT______sec
DATA POINTS______ FILTER______Hz
NO. SCANS/TRANSIENTS______

DECOUPLE GATED ☐
NUCLEUS ^{1}H GYROCODE

	1	2	3	4	5
A	☒	☒	☒	☐	☒
B	☐	☐	☐	☒	☐
C	☒	☐	☐	☐	☐
D	☒	☒	☐	☐	☐

OFFSET 51319 Hz RF POWER 119 dB
NOISE BANDWIDTH______Hz
INDOR SWEEP WIDTH______Hz
OPERATOR______ DATE APR 2 7 1978

CHART S-100XLA
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varian

APPENDIX E

METHYLATED NAPHTHALENES: ^1H (60 MHz) DATA

Chemical Shift Data in Delta for the Methyl Groups in the Polymethylated Naphthalenes

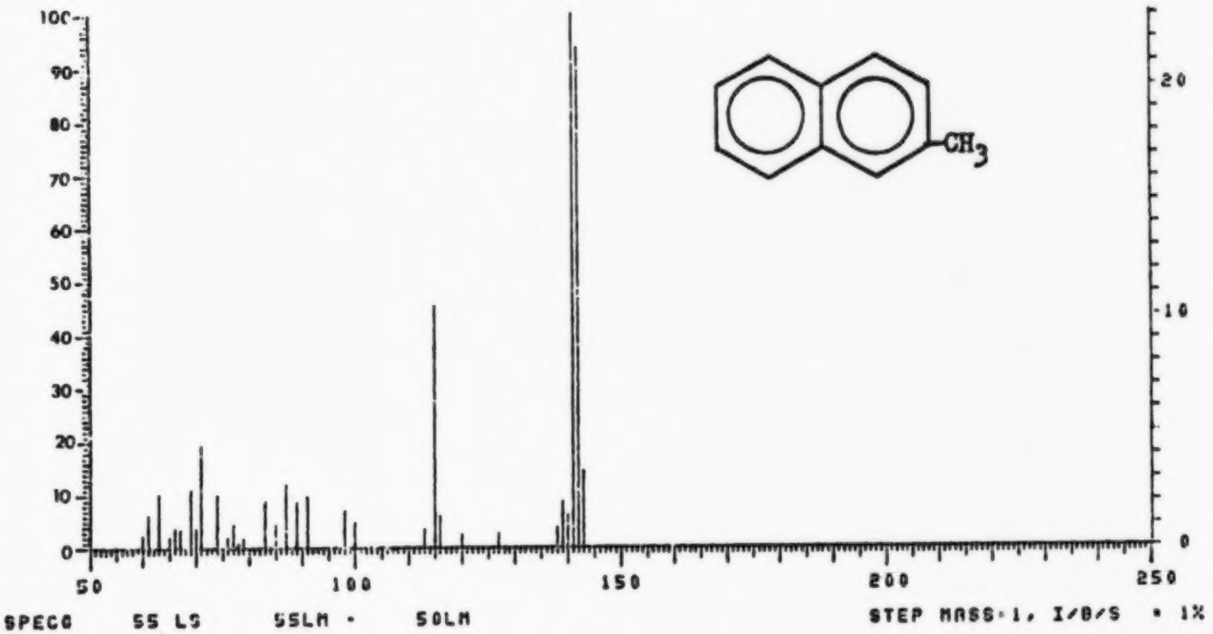
Compound	No. of Methyls*	δ Values**
3	1	2.50
9	3	2.73, 2.65, 2.59
10 & 10'	4	2.56, 2.56, 2.47, 2.43
11 & 11'	8	2.95, 2.95, 2.95, 2.95, 2.81, 2.68, 2.58, 2.45
12 & 12'	4	2.52, 2.52, 2.52, 2.52
13 & 13'	8	2.57, 2.57, 2.50, 2.50, 2.50, 2.50, 2.37, 2.37

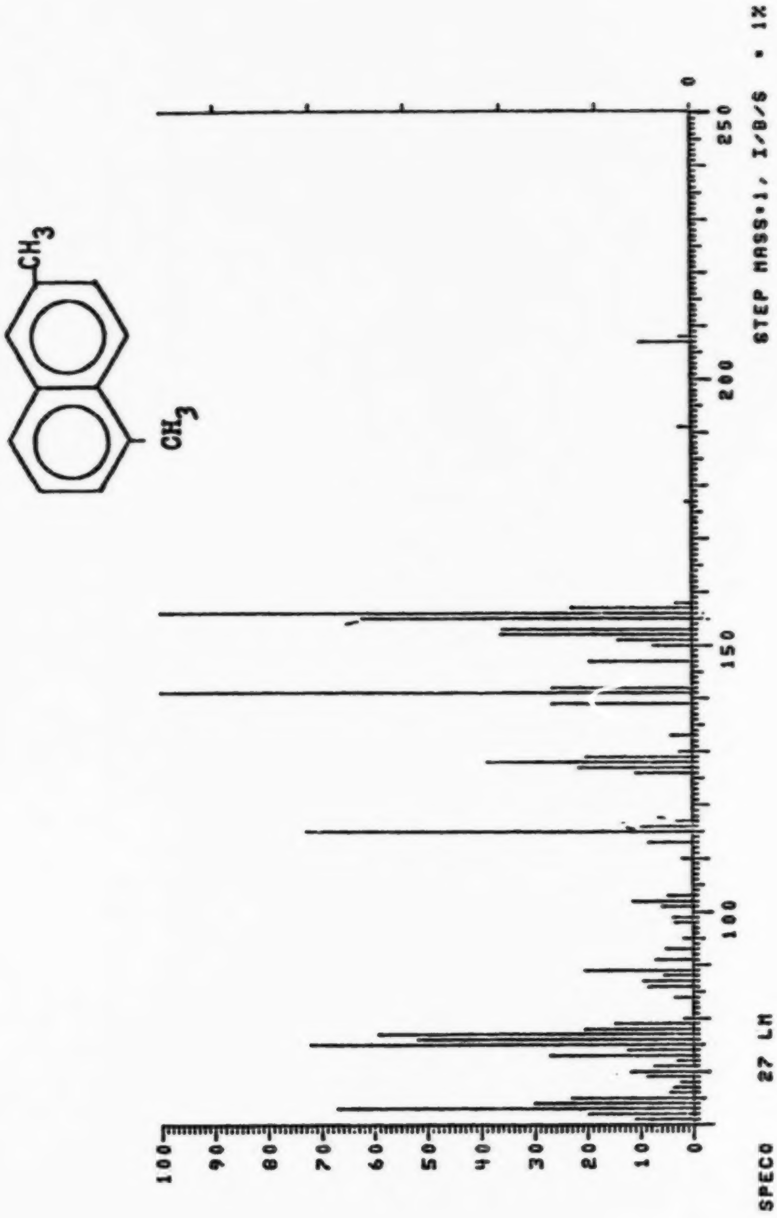
* All compounds exhibit correct CH_3/aromatic ratio by integration.

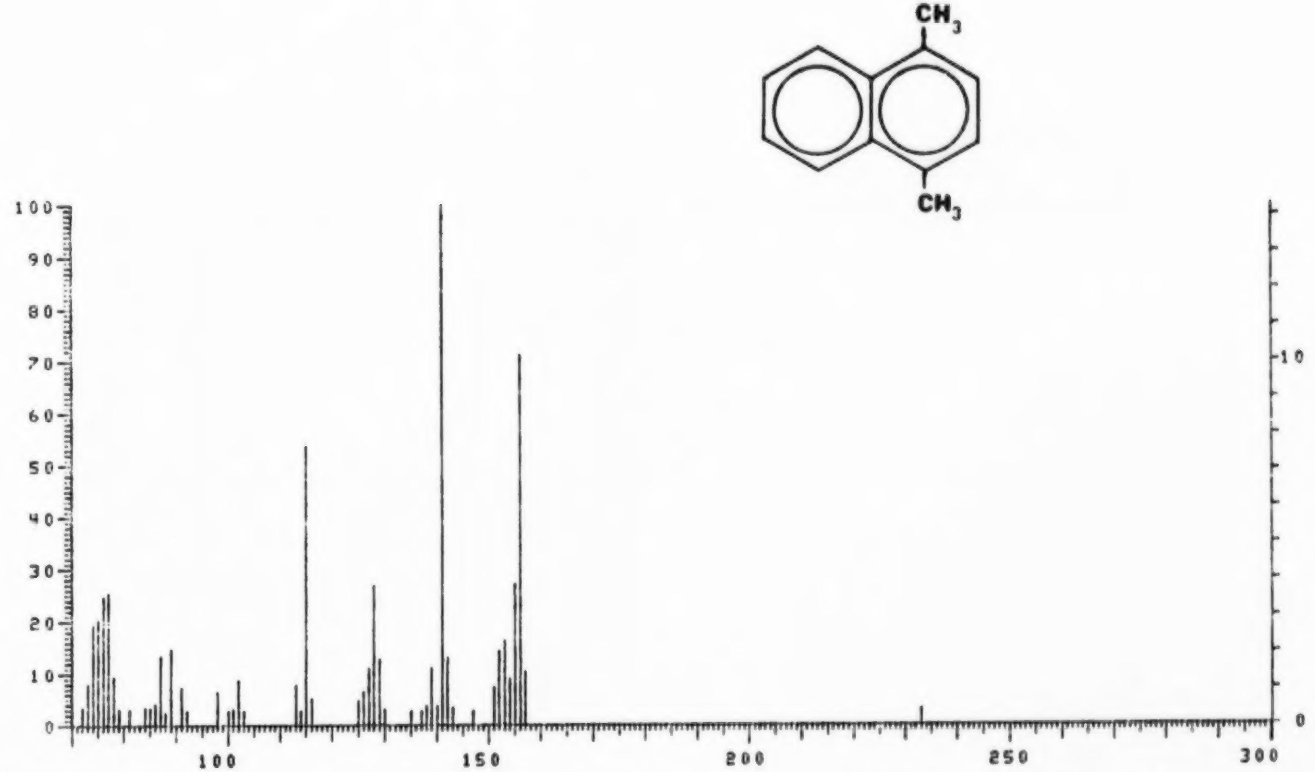
** Chemical shifts are relative to internal TMS.

APPENDIX F

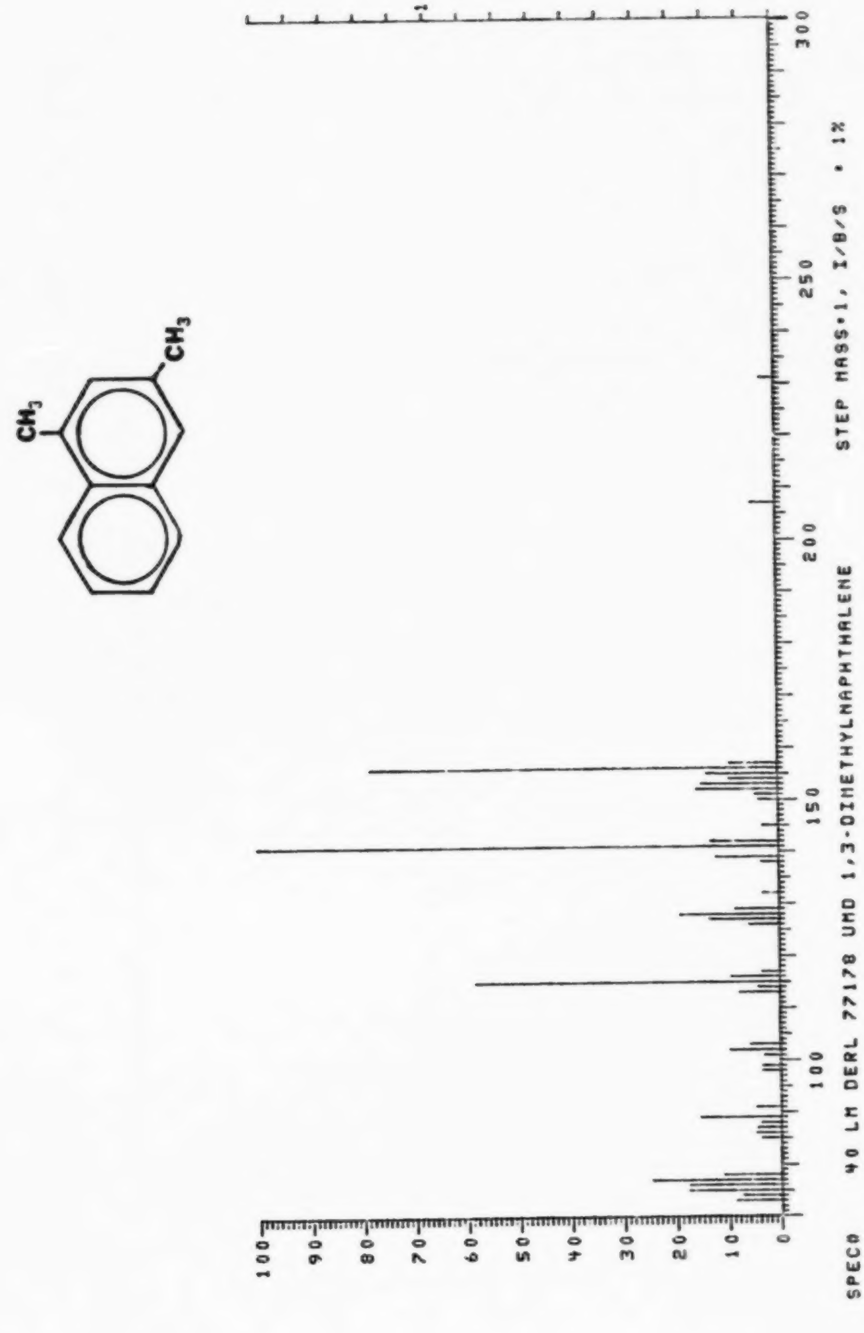
METHYLATED NAPHTHALENES: MASS SPECTRAL DATA FOR REPRESENTATIVE MONO-, DI-, TRI-, AND TETRAMETHYLNAPHTHALENES (SYNTHESIZED AND COMMERCIALLY AVAILABLE)

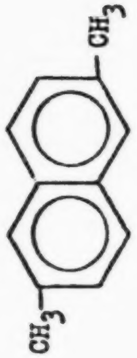


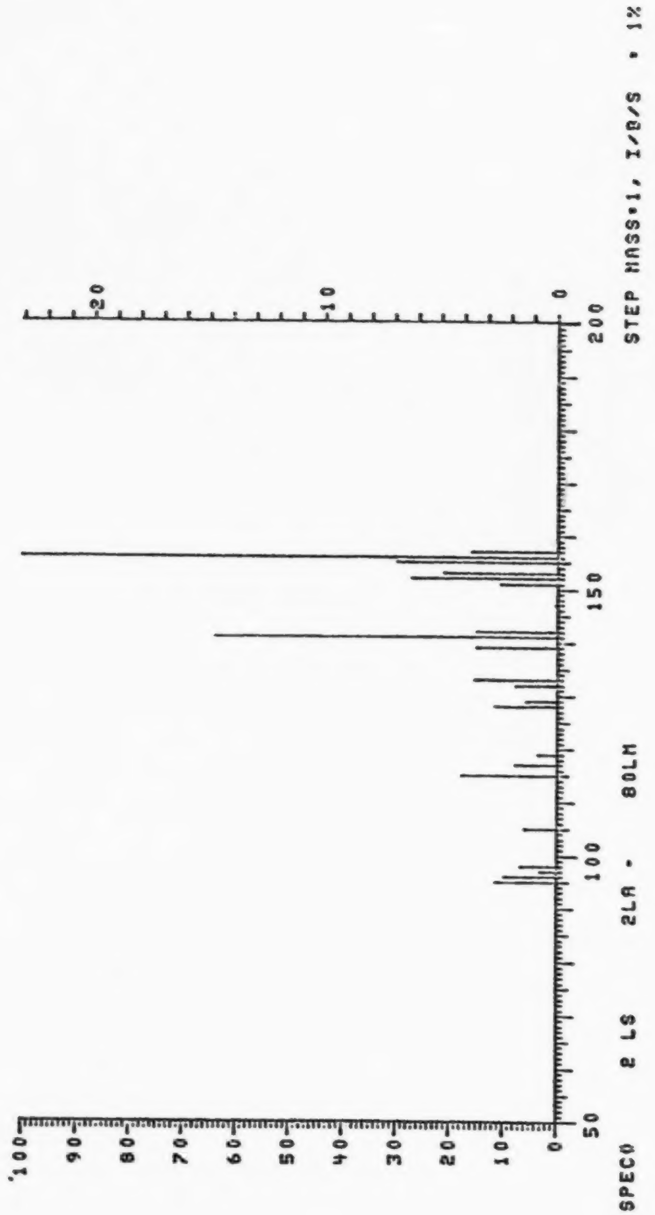

CH3
CH3
SPECO 27 LM
100
90
80
70
60
50
40
30
20
10
0
100
150
200
250
0
STEP MASS=1, I/B/S = 1%

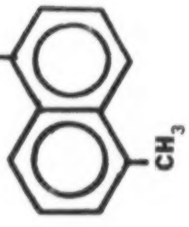


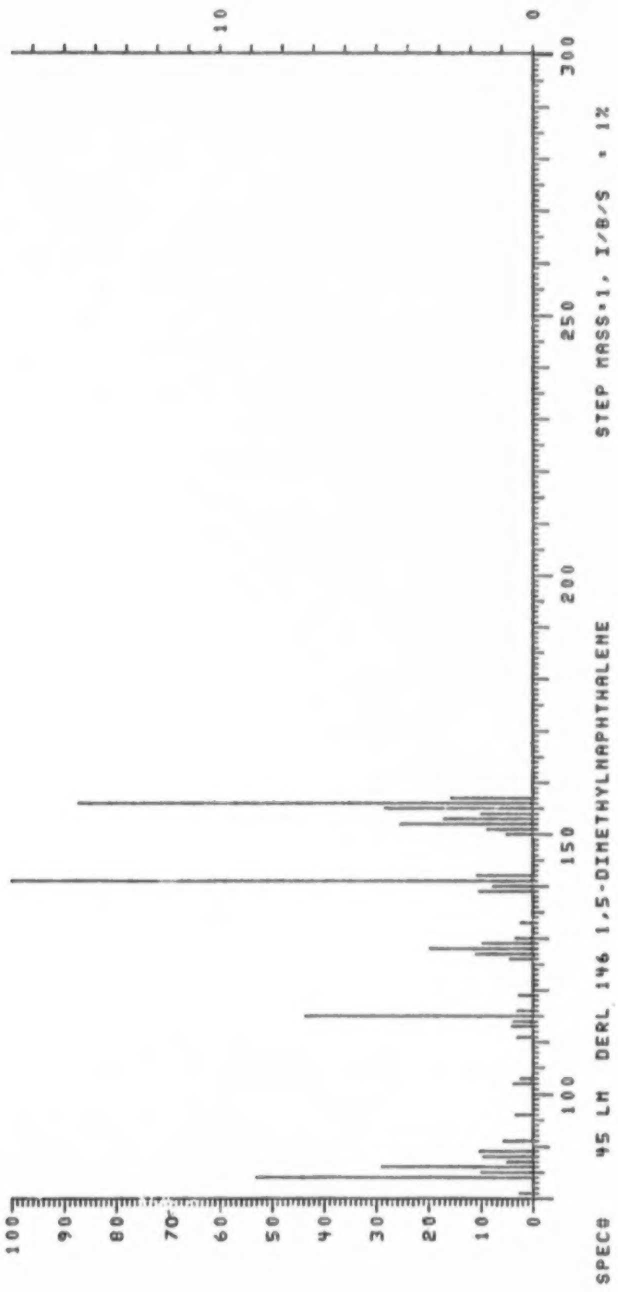
SPEC0 43 LM UMD OERL 77177 1,4-DIMETHYLNAPHTHALENE STEP MASS=1, I/B/S = 1%

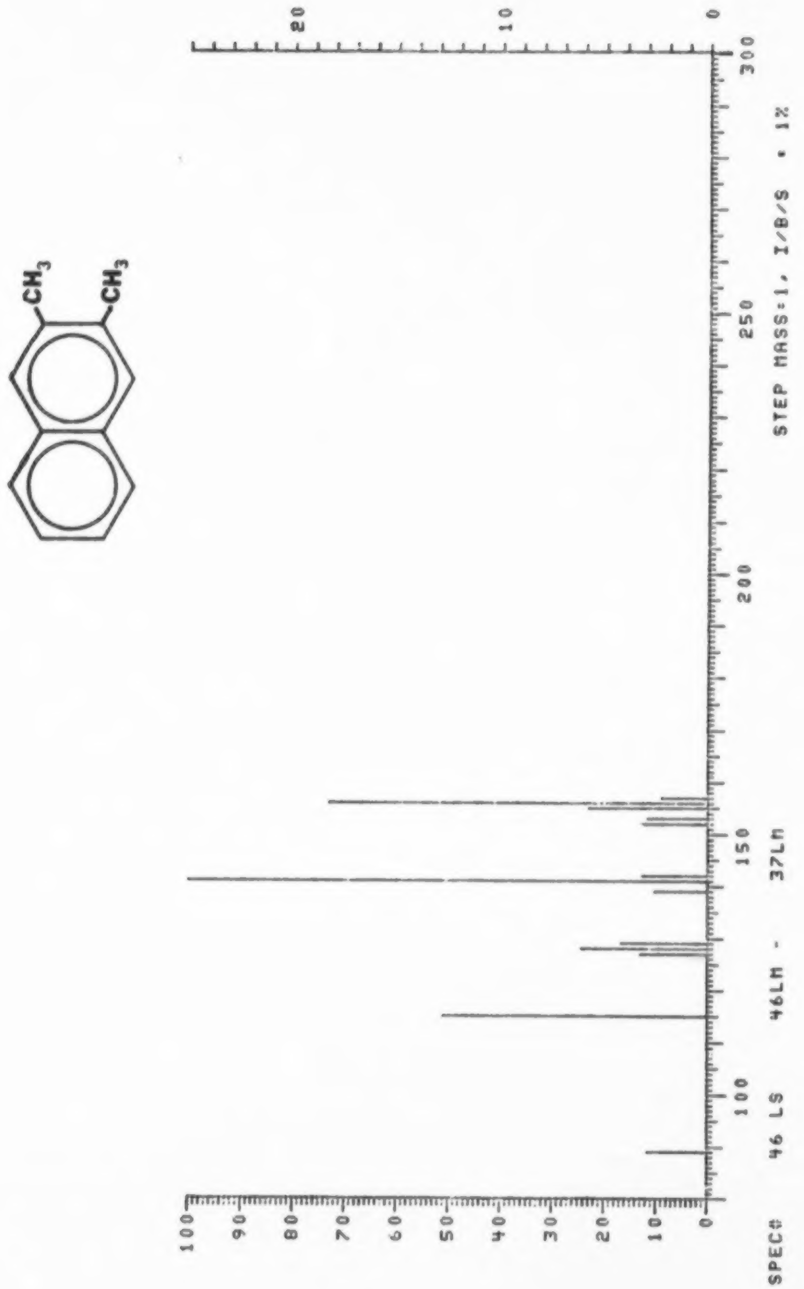

CH3
CH3
100
90
80
70
60
50
40
30
20
10
0
100
150
200
250
300
10
0
SPEC0 40 LM DERL 77178 UMD 1,3-DIMETHYLNAPHTHALENE STEP MASS=1, I/B/S = 1%

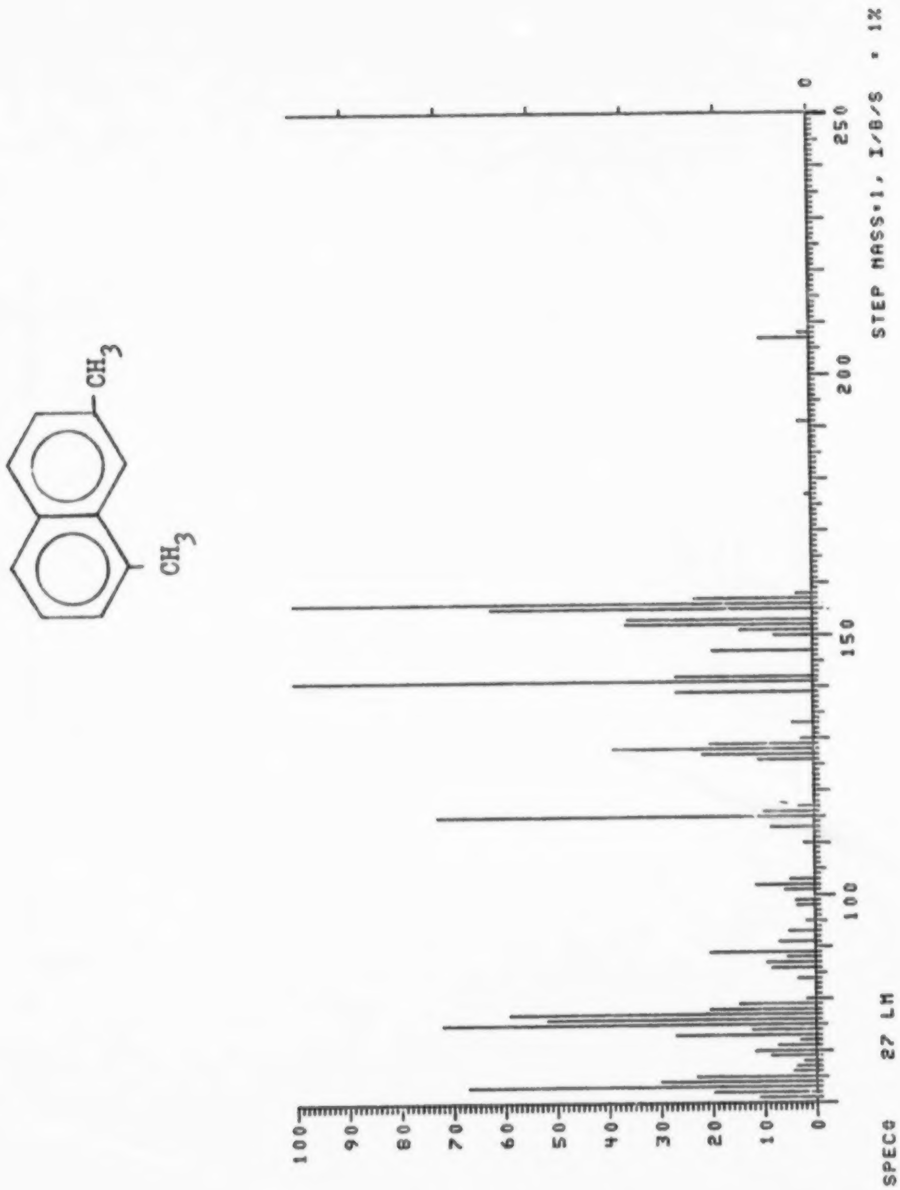

CH3
CH3

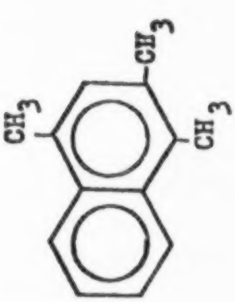

100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
20
10
0
SPECO 2 LS 2LA - 80LM
STEP MASS=1, I/B/S = 1%

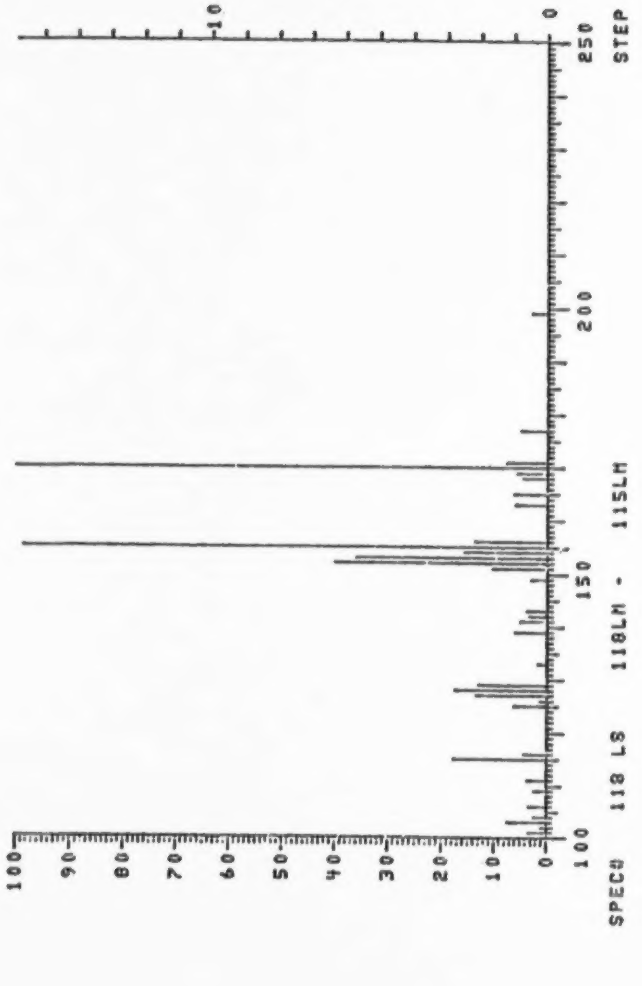

CH3
CH3

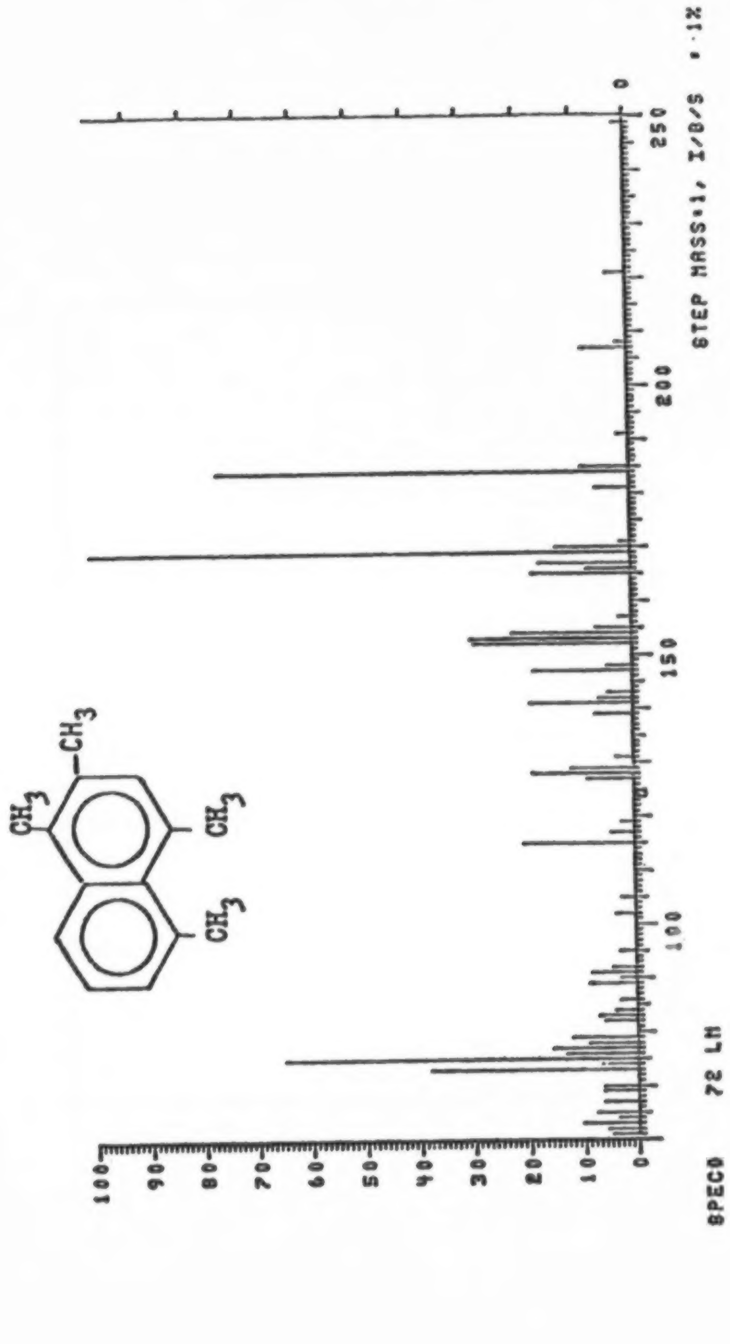

SPEC# 45 LM DERL 146 1,5-DIMETHYLNAPHTHALENE STEP MASS=1, I/B/S = 1%
100
90
80
70
60
50
40
30
20
10
0
100
150
200
250
300
10
0

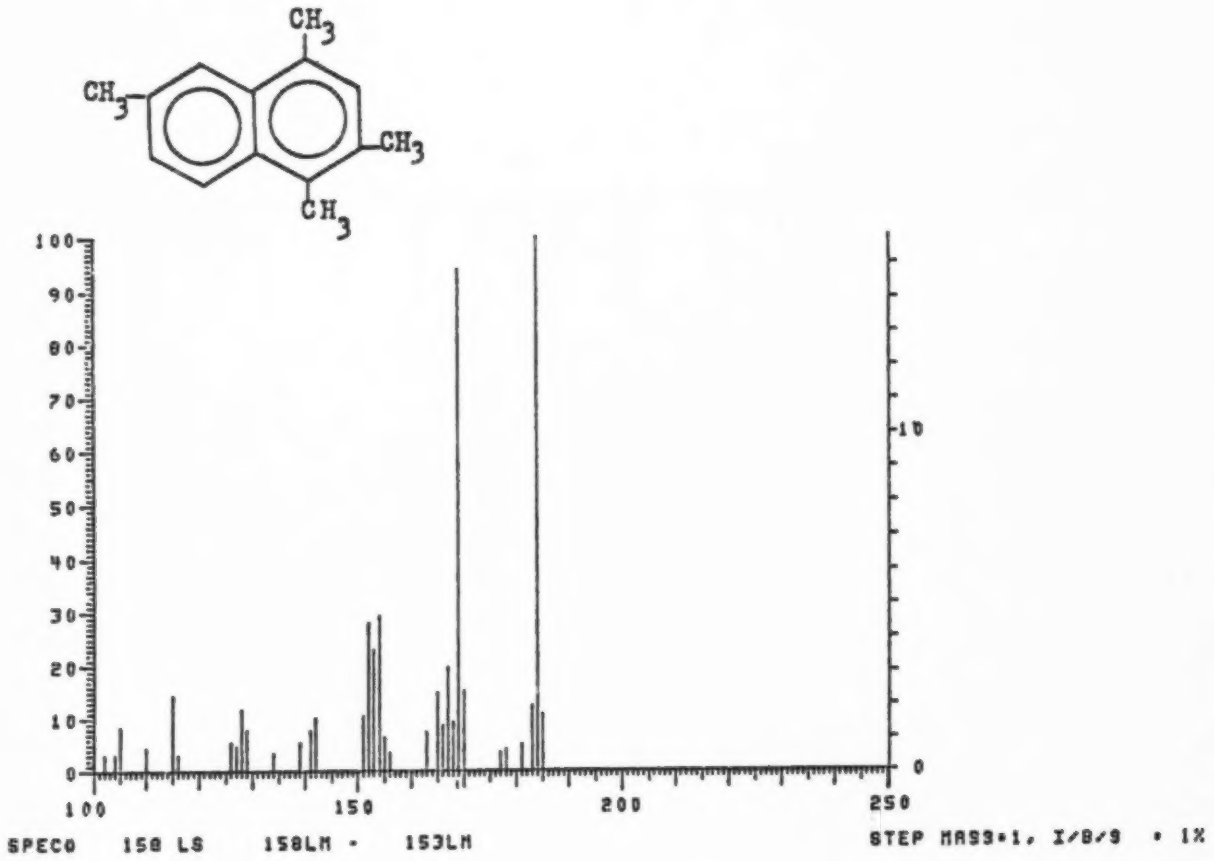

CH3
CH3
100
90
80
70
60
50
40
30
20
10
0
100
150
200
250
300
20
10
0
SPEC# 46 LS 46LM - 37LM STEP MASS:1, I/B/S = 1%


CH3
CH3
100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
0
SPEC0 27 LM
STEP MASS=1, I/B/S = 1%


CH3
CH3
CH3


100
90
80
70
60
50
40
30
20
10
0
100
150
200
250
-10
0
SPEC0 118 LS 118LM - 115LM
STEP MASS=1, I/B/S = 1%


SPECO 72 LM
STEP MASS=1, I/B/S = .1%
100
90
80
70
60
50
40
30
20
10
0
100
150
200
250
CH3
CH3
CH3
CH3


CH3
CH3
CH3
CH3
100
90
80
70
60
50
40
30
20
10
0
10
0
100
150
200
250
SPECO 150 LS 158LM - 153LM
STEP MASS=1, I/B/S = 1%

APPENDIX G

METHYLATED NAPHTHALENES: MICROANALYTICAL DATA

Microanalytical Data

Compound[1]	Calculated		Observed	
	%C	%H	%C	%H
3	92.51	7.67	92.46	7.54
	(82.46	7.55	82.48	7.57)
9	91.76	8.29	91.82	8.82
	(87.94	8.57	82.98	8.67)
10, 10'	92.26	7.74[2]		
	(82.94	8.57	82.79	8.20)
11, 11'	91.75	8.75	90.76	9.01
	(83.12	8.97	83.08	8.90)
12, 12'	91.25	8.66	91.23	8.75
	(83.12	8.97	83.10	8.91)
13, 13'	92.26	7.74	92.22	7.76
	(82.73	8.09	82.90	8.05)

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[1] Precursor alcohol in parentheses.

[2] Not enough pure material obtained.